

## REVIEW

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## The scent chemistry of butterflies

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Covering: 1990 up to 2022 Contrary to popular opinion, butterflies exhibit a rich chemistry and elaborate use of volatile compounds, especially for sexual communication, but also for defence. In contrast to night flying moths, in which commonly females are the producers of pheromones, male scent emission is prevalent in butterflies. While visual signals are generally important for long-range attraction, butterfly scent signals are often active only within a short range. Another feature of butterfly scent chemistry is the wide variety of compounds used, including alkaloids, terpenoids, fatty acid derivatives and aromatic compounds, sometimes with unique structures. This contrasts the structurally more restricted pheromone chemistry of moths. In this review, the compounds emitted predominately from male butterflies will be discussed and their ecological function explained, if known. The review includes material from 1990 to date, but will also cover older material to provide a necessary background.

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## 1 Introduction

The Lepidoptera, butterflies and moths, are one of the most species-diverse orders of insects and are intensively studied for their chemical communication. Most of the studies are centred on night-flying moth, due to their importance as pest insects. Obviously, chemical communication is of primary importance for an insect flying at night. Therefore, sex pheromones of thousands of moth species have been identified. They usually attract males to females over larger distances, making them preferred tools for pest control in agriculture. Chemically, moth

sex pheromones often belong to the Type I class, long-chain aliphatic compounds with 8 to 20 carbons, featuring double bonds at specific positions and with specific geometries, as well as a terminal alcohol, aldehyde, or acetate groups.<sup>1</sup> The second largest group, Type II, comprises polyunsaturated hydrocarbons, derived from linolenic or related polyunsaturated fatty acids, as well as epoxide derivatives, and the third group consists of the remaining compounds. The structures of moth pheromones, have been repeatedly reviewed<sup>1,2</sup> and a free database, Pherobase, exists where pheromone structures can be researched.<sup>3</sup>

In contrast, butterflies seemingly do not need to use pheromones, because their visual signals are so obvious. Nevertheless, mostly male butterflies have developed a multitude of diverse scent-disseminating structures in many families.<sup>4</sup> The compounds used are structurally more diverse than those of moths, originating from various biosynthetic pathways used by the butterflies or can be taken up from plants and modified. Their function can range from attraction to repellence, or function as defence, but butterfly pheromones are often used in short range during courtship. This makes testing compounds in ethological assays often quite difficult, so the actual function of many of the identified compounds is still unknown. A useful technique to access detection of volatiles by a receiving insect is electroantennography, meaning measuring the potential of an insect antennae or even single cells during chemical stimulation. When used together with GC in GC/EAD-setups, nerve impulses of complex mixtures can be recorded; however, this method gives no information of a potential behavioural or physiological response of the receiver. Nevertheless, the volatiles discussed here may have functions beyond pheromonal or signal activity. Physiological effects, *e.g.* by compounds

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| Family       | Subfamily     | Tribe       | Subtribe |
|--------------|---------------|-------------|----------|
| Nymphalidae  | Satyrinae     |             |          |
|              | Heliconiinae  | Heliconiini |          |
|              |               | Acraeini    |          |
|              | Danainae      | Danaini     |          |
|              |               | Ithomiini   | Olerina  |
| Lycanidae    |               |             |          |
| Pieridae     | Dismorphiinae |             |          |
|              | Coliathinae   |             |          |
|              | Pierinae      |             |          |
| Hesperiidae  |               |             |          |
| Papilionidae |               |             |          |

Fig. 1 Phylogenetic relationship of butterflies featured in the article according to Espeland *et al.*<sup>6</sup>

transferred during courtship, might be induced, or interaction with microorganisms might be affected,<sup>5</sup> but knowledge on these phenomena is currently scarce.

In this article, we focus on volatile compounds which are used by various species of butterflies of different families. The difficult phylogeny of butterflies has recently been revised,<sup>6</sup> leading to some differences compared to earlier conventions. The family *Danidae* was downgraded throughout the years to become now the tribe Danaini. We follow this modern phylogeny here, discussing compounds according to their biological origin. The different families, subfamilies, tribes and subtribes that are covered can be found in Fig. 1. The article sections are organized following roughly a historic perspective rather than the strict phylogenetic order for better understanding of the chemistry involved.

Some remarks may be necessary on the term volatile with respect to signals in insects. Even relatively large compounds

can be perceived by some insects through the gas phase, *e.g.* pentacosane, that attracts bumble bees from 1 m distance. This indicates that the term volatile includes many compounds not traditionally regarded as being volatile.

Throughout the years the methods for identifying butterfly natural products changed. While in the early phase isolation and NMR analysis dominated, requiring relatively large number of individuals, more modern methods include GC/MS analysis, microderivatization techniques and synthesis of the candidate, due to the usually low amount of material used, but also using metabolomic techniques. The respective identification techniques used can be found in the original literature; the synthesis of some compounds will be mentioned.

While a wide range of compounds, mostly produced by males, in contrast to moths, will be discussed, it might be helpful to the reader to compare these within the phylogeny. Therefore, compounds were colour coded in the figures with small dots. The colour code indicates occurrence only in a single genus (●), tribe (●), subfamily (●), or family (●).

## 2 Danaini

The Danaini, the milkweed butterflies, are a tribe of the subfamily Danainae of the Nymphalidae.<sup>6</sup> The males usually contain two types of scent glands, one of which is abdominal expandable brushes, so called hairpencils (HP), which are often used in hovering flights near to the female antennae to transport courtship pheromones.<sup>7</sup> Some species also use pheromone transfer particles, a form of dust produced by the HP, to transmit the chemicals. On the forewings a second type of scent organs can be found, androconia, which are male specific scent glands on the wings (Fig. 2). The butterflies need to bring the HP into contact with the androconia to produce courtship pheromones,<sup>8</sup> leading to the prediction of biosynthetic enzymes



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*Stefan Schulz studied chemistry at the University of Hamburg, Germany. He received his PhD under the guidance of Wittko Francke. In 1988, he took up a postdoctoral position with Jerrold Meinwald at Cornell University, Ithaca, USA, elucidating the biosynthesis of pheromones derived from pyrrolizidine alkaloids. After returning to Hamburg, he worked on his habilitation,*

*focusing on spider pheromones. In 1997, he became Full Professor of Organic Chemistry at the Technische Universität Braunschweig. His major research interests include Chemical Ecology and Natural Product Chemistry, aiming at the identification, synthesis, biosynthesis, and function of signaling compounds released by diverse organisms, including invertebrates, vertebrates and bacteria.*





Fig. 2 Expanded hairpencils and wing androconia of *Tirumala petiverana*. Reprinted by permission from John Wiley and Sons: *Biologie in unserer Zeit*, Pharmakophagie: Drogen, Sex und Schmetterlinge, M. Boppré, Copyright (2005).<sup>9</sup>

located in the androconia, although this has not yet been investigated.

The courtship behavior of the queen butterfly, *Danaus chrysippus*, has been investigated in detail in a landmark publication by Brower *et al.*<sup>10</sup> The major component of the courtship pheromone of many species is danaidone (**1**), the first butterfly pheromone identified, from *Lycorea ceres*.<sup>11–13</sup> Later, the related compounds danaidal (**2**) and hydroxydanaidal (**3**) were identified. Compound **3** is relatively widespread and has been detected in several genera such as *Amauris*, *Tirumala*, *Euploea*, *Ideopsis*, *Parantica* and *Danaus*,<sup>14–19</sup> while **2** is found only in *Danaus* and *Tirumala*.<sup>16–20</sup> The stereochemistry of **3** has been investigated in arctiid moths, where it is always (*R*).<sup>21</sup> A detailed account of the early work on the occurrence of these compounds and some related dihydropyrrolizines in butterflies has been published.<sup>21</sup> The dihydropyrrolizines are not restricted to danaines, but occur as well in ithomiines, another danaid tribe, and in arctiid moths, used similarly as courtship pheromones (Fig. 3).<sup>22</sup>

The dihydropyrrolizines **1–3** are biosynthetically derived from pyrrolizidine alkaloids (PAs).<sup>23</sup> Adult danaine males actively search for PAs, which can be found on withering plants.<sup>24</sup> The males then transfer the alkaloid onto the females during copulation, which in turn protects her eggs with PAs.<sup>25</sup> This transfer of male PAs into female eggs was mostly investigated in the arctiid moth *Utetheisa ornatrix*, which is easier to keep in the laboratory, but has also been demonstrated to exist in the queen butterfly, *Danaus gilippus*.<sup>25</sup> The females chose their mates according to the expected amount of PA, signalled

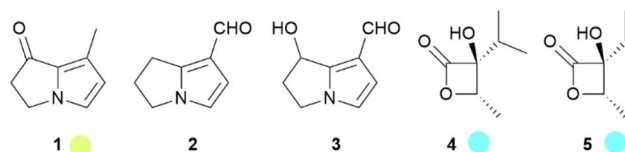


Fig. 3 Pyrrolizidine alkaloid derived dihydropyrrolizines and  $\beta$ -lactones. The colour code indicates occurrence only in a single tribe (●) or subfamily (●).

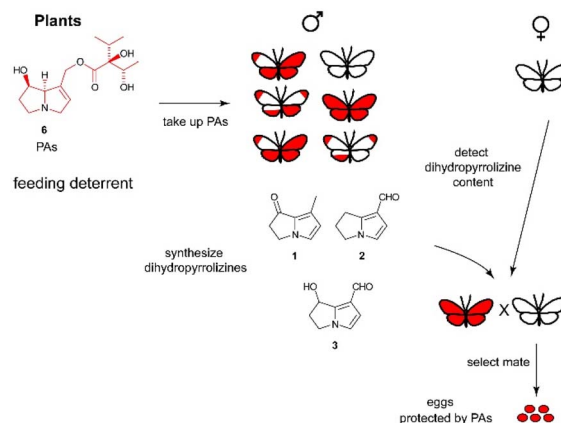


Fig. 4 Function of dihydropyrrolizines as courtship signal and indicator of PA load in danaines. Females obtain PAs from males, thus securing energy resources for other tasks.

by the pheromone concentration, obtained during mating. This ensures a high protection rate, because not every male is successful in PA sequestration. The whole PA-dependent pheromone system is shown schematically in Fig. 4 that also is similarly operative in ithomiine butterflies and some arctiid moths.<sup>25,26</sup>

The biosynthesis of **3**, the most widespread dihydropyrrolizine, has been investigated in males of the arctiid moth *Cretonotos transiens*.<sup>23</sup> A PA such as lycopsamine (**6**) is first cleaved into the free necine base retronecine (**7**). This base is then oxidized to the highly reactive diol **8**, which is oxidized to form hydroxydanaidal (**3**). As indicated in Fig. 5, **3** is the first relatively stable and sufficiently volatile compound in the reaction sequence, while all others are either too basic or too reactive to serve as a pheromone.<sup>26</sup>

Danaidone (**1**) has been synthesized originally by addition of methylpyrrol to acrylonitrile, followed by acidic cyclization,<sup>13</sup> but this approach has been improved.<sup>27</sup> Retronecine (**7**) can be obtained by hydrolysis of commercially available PAs such as monocrotaline. A subsequent oxidation with  $\text{MnO}_2$  delivers **3**.<sup>28</sup> Although most danaines contain dihydropyrrolizines in their HP, the Monarch butterfly (*Danaus chrysippus*) is a notable exception, even though adults readily visit PA containing plants as food source.<sup>29</sup>

The major components of the Monarch's hairpencil secretion<sup>29</sup> are (1*R*\*,3*S*\*,6*R*\*)-1,3,7,7-tetramethyl-2-oxabicyclo[4.4.0]dec-9-en-8-one (**9**),<sup>30,31</sup> benzyl hexanoate (**30**),<sup>29</sup> and (2*E*,6*E*)-3,7-dimethyldeca-2,6-dienedioic acid (**22**),<sup>29,32</sup> belonging to





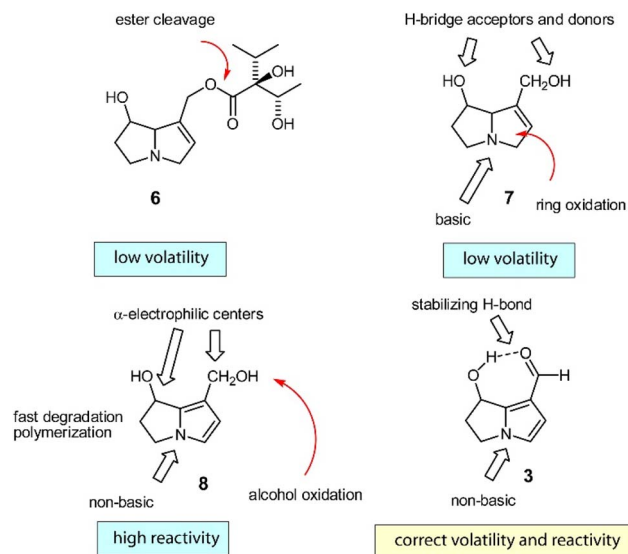


Fig. 5 Biosynthesis of hydroxydanaidal (3). Red arrows indicate metabolic changes. Reprinted by permission from Oxford Publishing Limited: Tiger Moths and Woolly Bears Behavior, Ecology, and Evolution of the Arctiidae, W. E. Conner, Copyright (2009).<sup>26</sup>

completely different compound classes. Ketone **9** is a  $C_{13}$ -terpenoid and is likely biosynthesized from carotenoids. Related apocarotenoids have been identified in other danaines, such as epoxytetrahydroedulan **10**, a major constituent of HP bouquets of various *Euploea* sp.,<sup>30,33</sup> and it has also been found in *Amauris hecate*.<sup>19</sup> Contrary to the dihydropyrrolizines, the presence of **10** is not dependent on the food sources of the adults. Lepidoptera usually take up carotenoids with their food, making it likely that the butterfly apocarotenoids are

biosynthesized from carotenoids ingested in the larval stage. Other apocarotenoids such as the putative biosynthetic precursors of **10**, dihydroedulans I and II (**11** and **12**), were found as minor components in danaines,<sup>19</sup> but are also known from plants. Nevertheless, none of the apocarotenoids were detected in the food plants of *E. mulciber*, supporting a *de novo* synthesis by the butterflies.<sup>33</sup> Compound **10** has been synthesized starting from enantioenriched dihydro- $\alpha$ -ionone, which was epoxidized, reduced, and cyclized to **12**, followed by epoxidation.<sup>30</sup> An enantioselective synthesis using (*S*)-2,4,4-trimethyl-2-cyclohexen-1-ol as starting material, was obtained by a lipase-mediated asymmetric hydrolysis. Chain elongation, diol formation, and cyclization delivered **12**, that was stereoselectively oxidized using a halohydrin intermediate.<sup>34</sup>

Another unique type of danaine HP compounds are bishomo-monoterpenes, or maybe more accurately, trisnorses-quitene. The male Monarch contains the hydroxy acid **21** and the diacid **22**,<sup>29,32,35</sup> while the Queen butterfly, *Danaus gilippus*, produces the related diol **20** (Fig. 6).<sup>36</sup> These compounds might be synthesized by the butterflies from farnesol (**29**) by oxidative loss of the terminal isopropyl-group.<sup>37</sup> *Euploea sylvestre* HPs contain the related compounds **23–25** showing a nerolidol-type head group.<sup>14</sup> The terminal dioxygenated motif common to these compounds is also found in related monoterpenes such as 8-hydroxy-6,7-dihydrogeraniol (**26**) and 8-oxogeraniol (**27**) of *Danaus chrysippus*.<sup>23,38</sup> Because **20** was not able to elicit response in electroantennographic recordings,<sup>39</sup> in contrast to **1**, it was postulated to serve as a glue for pheromone transfer particles of this species, enhancing longevity of the compound contact with the female antenna.<sup>12,39</sup>

Although a detailed analyses of the HP content of ten African danaine species of the genera *Amauris*, *Danaus*, and *Tirumala*

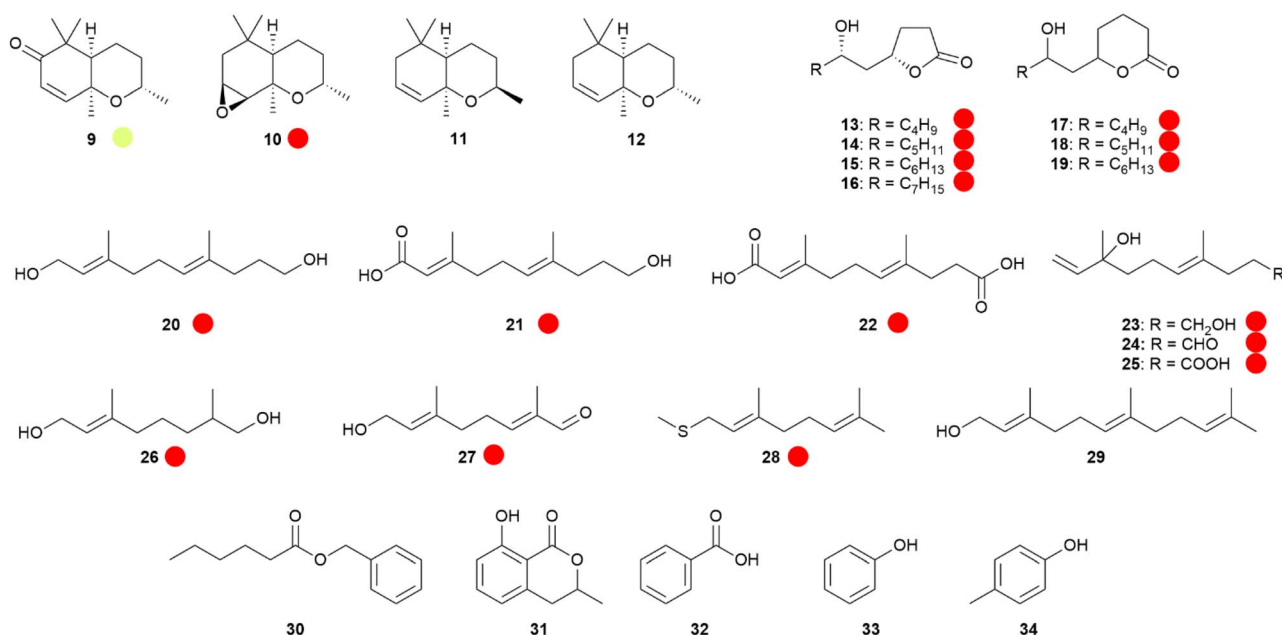


Fig. 6 Apocarotenoids and other terpenoids. Lactones and aromatic compounds of danaine hairpencils. The colour code indicates occurrence only in a single genus (•).

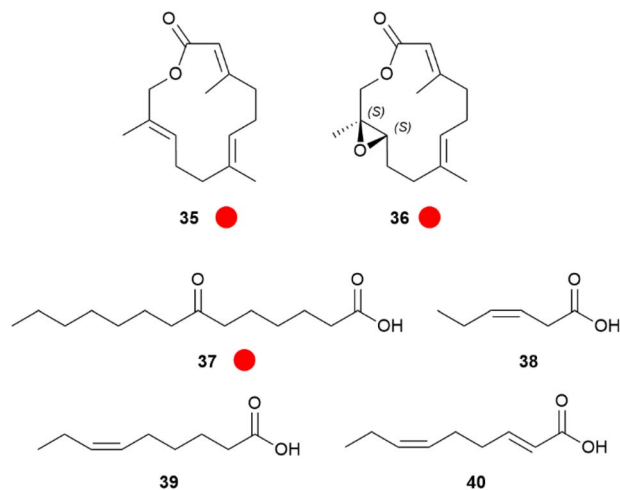


Fig. 7 Hairpencil components of *Amauris* butterflies. The colour code indicates occurrence only in a single genus (•).

showed the presence of 214 compounds in species specific mixtures with up to 59 components,<sup>19</sup> only **1** has a demonstrated function as aphrodisiac pheromone. This is largely due to the difficulty in performing bioassays with danaines, needing usually spacious flight cages for observation of behavior. Nevertheless, research on *Idea leuconoe* indicated that a larger share of the HP compounds are indeed active as semiochemicals. Semiochemicals comprise not only pheromones, which are active within a species, but also compounds that are used for interspecific chemical communication.

The analysis of the two types of HPs of *I. leuconoe* revealed the presence of **1**, but also of viridiflorine  $\beta$ -lactone (**4**) and its analogue **5**.<sup>40–42</sup> The latter are derived from the respective necic acids, occurring in PAs ingested this time during the larval stage<sup>43</sup> or modified by the butterfly.<sup>44</sup> Lactone **4** is also produced by *Euploea mulciber*.<sup>33</sup> In this species the males are only attracted to monoester PAs with correct necic base (7*R*)-stereochemistry such as intermedine or lycopsamine (**6**), but avoid diesters or (7*S*)-PAs like heliotrine.<sup>33</sup>

A blend of 15 compounds including geranyl thiomethyl ether (**28**), (*E,E*)-farnesol (**29**), the hydroxy- $\gamma$ -lactones **13–16**, phenol (**33**), *p*-cresol (**34**), benzoic acid (**32**), (*Z*)-9-tricosene, as well as **1** and **4** elicited strong searching as well as acceptance behavior by older females when applied to simple butterfly paper models.<sup>40</sup> This response was similar to that of an HP extract. Reducing the blend to just the PA derivatives **1** and **4** as well as **28** resulted in a weaker response, indicating a lower efficacy. Danaidone (**1**) alone induced the weakest response. All compounds elicited responses in EAG experiments in both males and females, thus forming a pheromone bouquet.<sup>40</sup> These results indicate that the many compounds found on HPs play an important semiochemical role and are behaviourally active, acting at least as pheromonal synergists.

The thiomethyl ether **29** is one of the rare cases of an insect pheromone component containing sulphur. It is so far only found in *I. leuconoe*, as are the hydroxy- $\gamma$ -lactones **13–16**. These lactones, and similar hydroxy- $\delta$ -lactones, occur as minor

components, and were synthesized using enantioselective reductions of 4,6- or 5,7-dioxoalkanoates by Noyori's Ru-BINAP catalysts.<sup>42,45</sup> The absolute configuration of these lactones in the HP has been determined.<sup>42,45</sup> While the major hydroxy- $\gamma$ -lactone 6-hydroxy-4-undecanolide (**14**) exclusively occurs as the (4*S*,6*S*)-enantiomer,<sup>45</sup> surprisingly all four enantiomers of the hydroxy- $\delta$ -lactones are present in the HP. For example, 7-hydroxy-5-dodecanolide (**18**) is found as a 92:8 *anti* (5*S*,7*S*/5*R*,7*R* 7:3): *syn* (racemic)-mixture. These values differ for the congeners **17** and **19**.<sup>42</sup> In summary, the enzymes responsible for the lactone biosynthesis seem to be tuned to the formation of the  $\gamma$ -lactones, while the  $\delta$ -lactones are side products with lower stereoselectivity within the required biosynthetic transformations.

While the concentration of compounds **1**, **4**, **14** and (*Z*)-9-tricosene in the HPs increased with age, the presence of the fungal metabolite mellein (**31**) depended on access to itself. Mellein (**31**) serves as phagostimulant for *I. leuconoe*, as does methyl *p*-hydroxybenzoate.<sup>46</sup> Both compounds are phenols, as are the HP compounds **33** and **34**.

The extensive research on *I. leuconoe* showed that a variety of compounds can have a behaviour-modifying effect on the butterflies. These compounds originate from a variety of sources, some are taken up during feeding or pharmacophagously (**1**, **4**, **31**) and were modified, while others are synthesized by the butterfly using the fatty acid (**14**) or terpene biosynthetic pathways (**28**).

Larger lactones are found in HPs of the African *Amauris niavius*.<sup>47</sup> The two major components of the secretion, niaviolide (**35**) and epoxyniaviolide (**36**), were the first natural macrocyclic sesquiterpenes (Fig. 7). They represent terminally oxidized sesquiterpenes that are cyclized. Generally, lactones are well suited to serve as volatile signals, because a putative hydroxyacid precursor with low volatility can be stored and then converted into the much more volatile lactone by simple esterification when needed.<sup>47,48</sup> Both lactones were synthesized starting from methyl farnesenoate by terminal  $\text{SeO}_2$  oxidation and ring closure with or without a prior Sharpless epoxidation.<sup>47</sup> Biosynthetically, similarly to **10**, a terminal epoxidation is used to produce a singular specific danaine compound. Other major HP compounds are again **1** and 3,4-dimethoxyacetophenone (**50**).<sup>19,49</sup>

Common fatty acids such as palmitic, stearic, oleic, linoleic or linolenic acids are often found in HP extracts, mostly in low concentration. It remains unclear whether they are true glandular constituents or degradation products liberated from lipids during sample preparation. Nevertheless, some *Amauris*

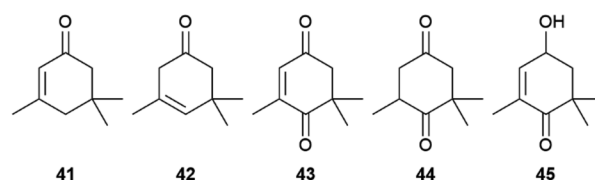
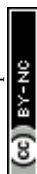


Fig. 8  $\text{C}_9$ -Apocarotenoids of *Amauris echeria* and *Euploea sylvester*.



spp. contain unique fatty acids. A whole range of 63 fatty acids ranging from hexanoic acid to docosatrienoic acid were identified in *A. echeria*, *hecate*, *albimaculata*,<sup>50</sup> and *ochlea* HP extracts.<sup>50,51</sup> Of special interest were several oxidized acids of *A. echeria* including (*E*)-7-oxo-11-tetradecanoic acid (37), as major constituent, accompanied by the (*Z*)-isomer, saturated and shorter analogues. *A. ochlea* HPs contained predominately hexanoic, (*Z*)-3-hexenoic (38), nonanoic, (*Z*)-6-nonenoic (39), as well as minor amounts of (2*E*,6*Z*)-nona-2,6-dienoic acids (40).<sup>50</sup>

Next to these acids, 1, 3, and several other apocarotenoids were identified in *A. echeria*, but of the C<sub>9</sub>-type instead of the C<sub>13</sub>-type discussed above. Major components were oxoisophorone (43) and its hydrogenated analogue 44, as well as the parent phorones 41 and 42 (Fig. 8). The same apocarotenoids were also present in *Euploea sylvestris*, with the reduced 45 as major component.<sup>50</sup> Interestingly, 43 is also the major volatile attracting butterflies to the invasive butterfly bush *Buddleja davidii*,<sup>52</sup> indicating a close relationship between flower attractants and butterfly signalling compounds.

Several aromatic compounds such as vanillin (49), eugenol (51), methyl salicylate (48), benzaldehyde (46) or anisole (47) are frequently found in danaine HPs, but not always in the same species (Fig. 9). While a compound can be a major component of a HP compound bouquet, it may be a minor component in another species.<sup>50</sup> A comparative analysis of African milkweed butterflies including 10 species of the genera *Danaus*, *Tirumala* and *Amauris* listed 214 identified compounds,<sup>19</sup> including non-volatile compounds such as long-chain 2,5-dialkyltetrahydrofurans (THFs).<sup>53</sup> The latter are typical constituents of epicuticular lipids of sun-exposed Lepidoptera.<sup>54</sup> THFs occur together with long-chain linear alkanes and alkenes in the epicuticle of insects. Nevertheless, some species such as *A. niavius* contain large amounts of still volatile shorter hydrocarbons such as heneicosane and heneicosene.<sup>19</sup> Such compounds are known as pheromones from some pierids (see pierid section).

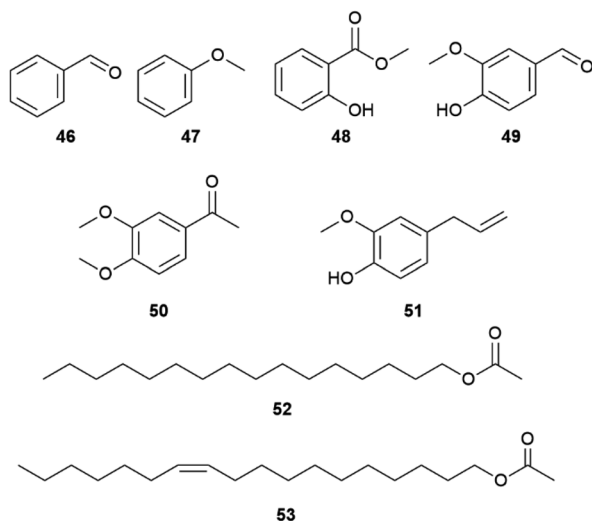


Fig. 9 Aromatic compounds and aliphatic esters from danaines.

Of special interest is also the occurrence of linear terminal esters, hexadecyl (52) and vaccenyl acetates (53) that, together with 1, represent the major HP constituents of *Lycorea ceres ceres*, a phylogenetically basal danaine.<sup>55</sup> The similarity to the type I moth pheromones, linear aliphatic unsaturated alcohols, acetates or aldehydes,<sup>1</sup> indicate these compounds to be a primal trait that seems to be lost in more recent species. Interestingly, the  $\Delta$ 11-desaturase needed for the formation of 53 is the most important desaturase for moth pheromone biosynthesis.<sup>56</sup>

The composition of the HP secretion of danaines have been used for a cladistics analysis of the phylogeny of the danaines, using 68 volatile compounds, but excluding dihydropyrrolizines.<sup>57</sup> A combination with morphological data revealed better phylogenetic solutions for African milkweed butterflies than purely morphological methods, but genetic methods were not available at that time. Nevertheless, the results indicate a phylogenetic dependence of the HP composition.

### 3 Ithomiini

The ithomiines, also known as glass-wing butterflies due to their sometimes transparent wings, are a sister subfamily of the danaines, occurring with high species diversity in Central and South America. In contrast to the danaines, the ithomiines lack abdominal scent brushes, but feature instead erectable hairs on the wings that are used to disseminate volatile compounds during courtship (Fig. 10).

As with the danaines, males are attracted to PAs,<sup>24,59</sup> feeding on them to protect themselves and to convert them into aphrodisiac pheromones, as with the danaines. Lycopsamine (6) is the primary PA stored in ithomiines,<sup>60</sup> and PAs with different side chain stereochemistry are converted into 6, at least in *Mechanitis polymnia*.<sup>61</sup> Ithomiines also use PA-derivatives such as danaidal (2) and hydroxydanaidal (3) in their androconia.<sup>54</sup> Thirteen genera of 30 investigated contained



Fig. 10 Male *Oleria onega janarilla* with erected scent brushes (androconia). The picture is reproduced from Beilstein Journal of Organic Chemistry, F. Mann et al., under a CC-BY-4.0 licence.<sup>58</sup>



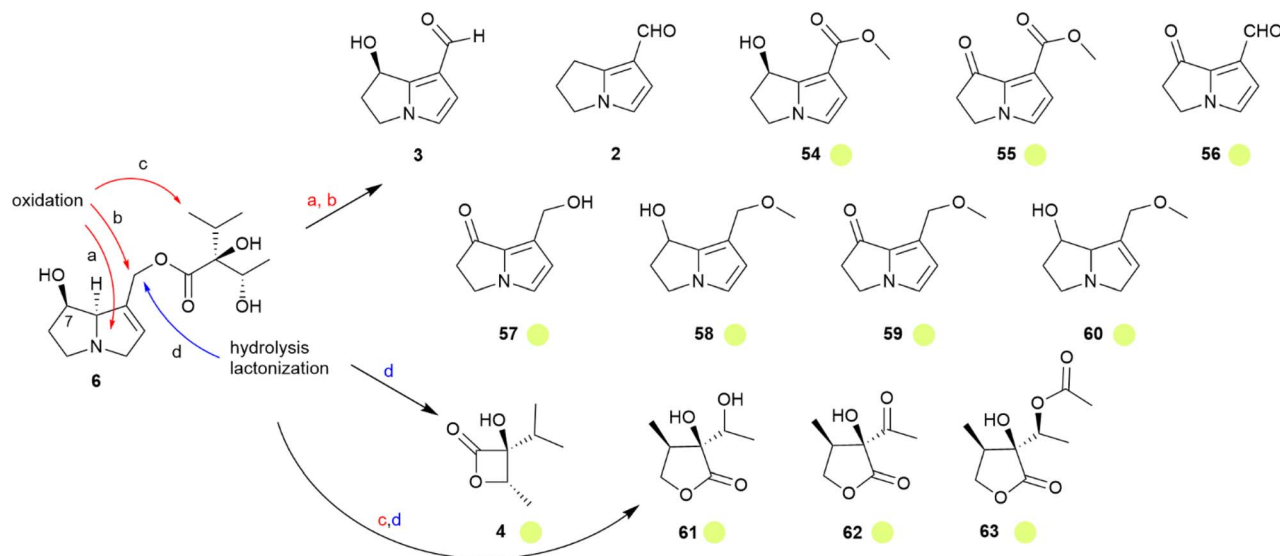


Fig. 11 Transformations of lycopsamine (6) into volatile dehydropyrrolizines and necic acid lactones, adopted from Schulz *et al.*<sup>54</sup> The colour code indicates occurrence only in tribe (•). Reprinted by permission from Elsevier: *Biochemical Systematics and Ecology*, Semicomicals derived from pyrrolizidine alkaloids in male ithomiine butterflies (Lepidoptera: Nymphalidae: Ithomiinae), S. Schulz, Copyright (2004).<sup>54</sup>

these derivatives.<sup>54</sup> While **1** was not found, an ithomiine specific major compound, present in seven genera, is methyl hydroxydanaidoate (**54**).<sup>14,54</sup> Both **3** and **54** usually dominate the androconial secretion, but minor amounts of the oxidized or methylated derivatives **55–60** were present in few species.<sup>54</sup> The necic acid derived  $\beta$ -viridiflorine lactone (**4**), known from some danaines, was detected in *Melinaea menophilus* and *Scada kusa*.<sup>54</sup> Unique for ithomiines is the  $\gamma$ -lactone ithomiolide A (**61**).<sup>14,62</sup> Its absolute configuration has been established by stereoselective synthesis starting from (*S*)-3-methylbutyrolactone,  $\alpha$ -ethenylidenation and ketol formation, followed by reduction to the target diol. Trace compounds of ithomiolide B (**62**) were also detected, as well as the acylated metabolite **63**.<sup>54,63</sup> The stereochemistry of **61** is identical to that of (–)-viridifloric acid, the necic acid of **6**.<sup>63</sup> Therefore, the PA content of the male butterflies is adjusted to give a large pool of the proper alkaloid **6**, which is in turn in part transformed into the various dihydropyrrolizine or lactone compounds, as shown in Fig. 11. Interestingly, **61** was detected in eleven genera, all of them belonging to a clade of more advanced genera.<sup>54</sup> A comparison of the data using a recent phylogeny supports the initial hypothesis.<sup>64</sup> Ithomiolides are formed within a clade containing the subtribes Ithomiina, Napeogenina, Dircennina, and Godrydina, although in the Oleriina no volatile dihydropyrrolizines or lactones were detected at all.<sup>65</sup>

The formation of dihydropyrrolizines from PA requires various biosynthetic transformations, mostly oxidative in nature (Fig. 11). While dihydropyrrolizine biosynthesis has been discussed within the danaine section, the formation of ithomiolides is unique. Their biosynthesis requires an oxidation of an unactivated methyl group (c in Fig. 11) because no PA with terminally oxidized isopropyl groups have been reported from food plants. It seems likely that the advanced ithomiines use a more specific signal such as **61**, generated by this oxidative

transformation. This avoids confusion with widely used dihydropyrrolizines such as hydroxydanaidal (**3**). The latter compound has been claimed to be formed not only by Lepidoptera, but also by degradation of PA-containing plants, attracting various Lepidoptera over longer distances.<sup>66</sup>

The absence of PA derivatives in Oleriina, including the genera *Oleria* and *Hyposcada*, has been repeatedly shown, leading to a detailed analysis of *Oleria onega*.<sup>67</sup> In the androconia only the alkaloid **4** was detected, accompanied by a quite unstable degradation product, dehydropyrrolizine **64**. This compound was synthesized from carboxymethylpyrrolizine by reduction and elimination.<sup>67</sup> Compound **64** is likely a degradation product formed within the injection port of the gas

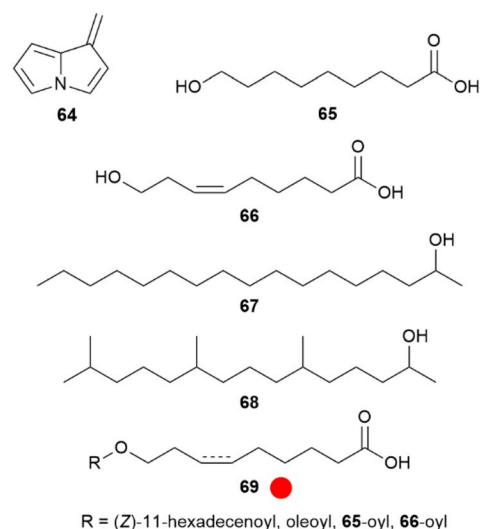


Fig. 12 Compounds of androconia of *Oleria onega*. The colour code indicates occurrence only in a single genus (•).





chromatograph, but formation during PA degradation occurring in plants cannot be ruled out. Nevertheless, major volatile compounds of the androconia proved to be the acids 9-hydroxynonanoic (**65**) and (*Z*)-9-hydroxy-6-nonenoic (**66**) acids, as well as 2-heptadecanol (**67**) and 6,10,14-trimethyl-2-pentadecanol (**68**) (Fig. 12).

Although not volatile, esters (**69**) of both **65** and **66** with predominately (*Z*)-11-hexadecenoic acid constitute a major part of the secretion, maybe serving as a fixative, or as storage or a slow release form of the two acids. Similarly, acids of type **69** can also be formed by conjugation between acids **65** and **66** themselves.<sup>67</sup> Such ester acids are not reported from other organisms. Compounds **65**, **66**, and esters **69** were synthesized using classic alkyne or Wittig approaches, proper silyl-protection and esterification using EDC.<sup>67</sup>

Another type of compound was detected in *Ithomia salapia*, prenyl esters. While **61** was identified in *I. s. derasa*, the subspecies *I. s. aquinia* contained the sesquiterpene  $\alpha$ -elemol (**70**).<sup>58</sup> Some side products indicate that potentially hedycaryol (**71**) is the actual sesquiterpene present that undergoes Cope rearrangement in the heating block of the GC/MS system during injection.<sup>58</sup> Nevertheless, rearrangement induced by heat or sun within the androconia cannot be excluded. The difference in the composition of the volatile compounds may explain the low gene flow between the two species even in regions where they co-occur.<sup>58</sup>

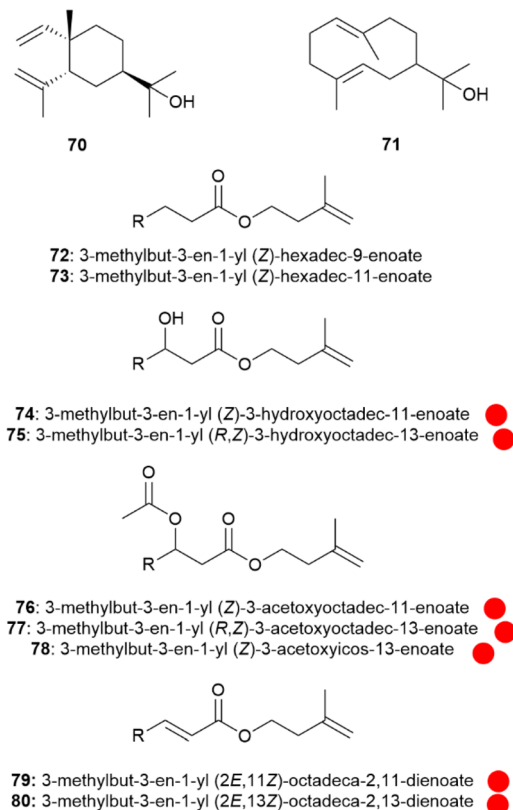


Fig. 13 Terpenoids and isoprenyl esters of *Ithomia salapia*. The colour code indicates occurrence only in a single genus (●).

Major constituents of the androconia are a group of 3-methyl-3-butenyl (isoprenyl) esters of fatty acids (Fig. 13).<sup>58</sup> These fatty acids comprise unsaturated compounds with  $\omega$ -7 and  $\omega$ -9- double bonds, as well as 3-hydroxy and 3-acetoxy acids. The absolute configuration of the major parent acid, (*Z*)-3-hydroxy-13-octadecenoic acid was determined to be (*R*).<sup>58</sup> Surprisingly, most of the esters (**72–80** are shown here as examples), occur as a mixture of two isomers differing in the position of the double bond, e.g. isoprenyl (*Z*)-3-acetoxy-11-octadecenoate (**76**) and isoprenyl (*Z*)-3-acetoxy-13-octadecenoate (**77**). The formation of these isomers can be explained by activity of both a  $\Delta$ 9-and a  $\Delta$ 11-desaturase, likely introducing the double bonds after fatty acid biosynthesis. The  $\Delta$ 11-desaturase is the typical enzyme used in moth sex pheromone biosynthesis.<sup>68</sup> Although the specific function of compounds **72–80** are not clear, an involvement in signalling of *Ithomia salapia* is highly likely, due to the unique structures and involved enzymes.

## 4 Heliconiinae

The Heliconiini, the passion-vine butterflies, are a tribe of the subfamily Heliconiinae of the Nymphalidae.<sup>6</sup> The genus *Heliconius* is particularly known for its high species diversity and participation in Müllerian mimicry and therefore serves as a model for evolutionary studies.<sup>69,70</sup> The males exhibit two types of scent glands, abdominal clasper scent glands (CSG, Fig. 14D), which are used to transfer antiaphrodisiacs (AA) together with a complex mixture of compounds during

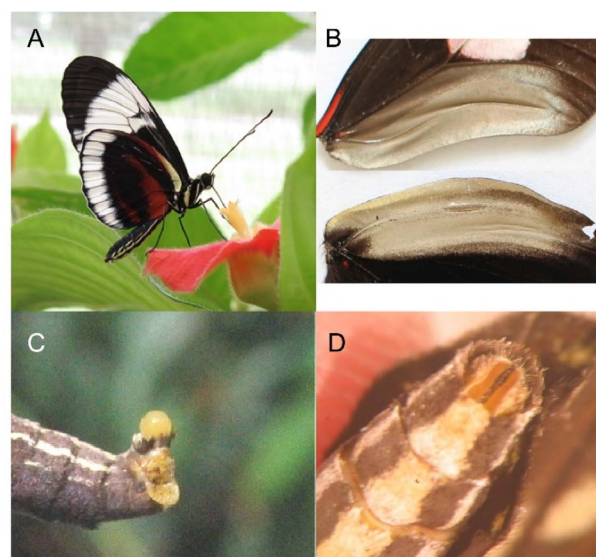
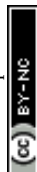


Fig. 14 *Heliconius cydno* (A),<sup>77</sup> expanded view of male forewing and hindwing androconial region (B),<sup>75</sup> female abdominal gland (C) and male clasper scent glands (D) of *H. charitonia*.<sup>78</sup> The picture 14A is reproduced from Ecology and Evolution, K. J. R. P. Byers *et al.*,<sup>77</sup> and picture 14B from Peer Journal, K. Darragh *et al.*,<sup>75</sup> under a CC-BY-4.0 licence. Pictures 14C and 14D are reprinted by permission from John Wiley and Sons: Evolution, Sexual selection drives the evolution of antiaphrodisiac pheromones in butterflies, C. Estrada *et al.*, Copyright (2011).<sup>78</sup>





copulation to the abdominal pouch of the female (Fig. 14C). The AA lead to a reduced attractiveness of the female for courtship with other males and ensuring undisturbed egg-laying, although additional functions may also be possible, e.g. defensive display.<sup>71–73</sup> The other male specific scent glands are the androconia, which are specialised brush-like scales in the overlapping region of the fore and hind wing with a distinct greyish sheen (Fig. 14B).<sup>74,75</sup> The androconial scales release volatiles that might act as pheromones during courtship by androconial exposure, which occurs in every courtship that results in mating, and they are involved in female mate choice.<sup>74,76</sup>

#### 4.1 Clasper scent glands

*Heliconius* butterflies are divided into two clades, one is polyandrous (58%) where females mate with several males within a breeding season, while the other is monoandrous and displays a unique mating behaviour known as pupal-mating.<sup>79</sup> In such species males search for immatures, guard them and wait until they are able to mate with the emerging female.<sup>74,80</sup> Important for this pre-copulatory mate guarding is the ability of males to recognize immature conspecific females and estimate their maturity.<sup>81</sup> *H. charithonia* males can recognize female pupae using sex specific volatile monoterpenes, which are released towards the end of pupal development.<sup>80</sup> While the main components of the pupal cuticle are typical cuticular lipids such as saturated hydrocarbons or 2,5-dialkyltetrahydrofurans, in late pupae some monoterpenes could be observed by SPME analysis of living pupae. Whereas the monoterpene linalool (**81**) exclusively occurs in late male pupae, linalool oxide (furanoid, **82**) was released by late female pupae. Together with linalool oxide acetate (**83**), present in both sexes, these monoterpenes were specific for late pupae (Fig. 15). Bioassays showed that males are attracted to female specific **82**, while they are repelled by male specific **81**, the former occurring as a mixture of all four stereoisomers in the female pupae.<sup>80</sup> Other, nonspecific volatiles emitted by early stages of the pupae were cyclohexanol and its acetate,  $\alpha$ -pinene, and **11**.<sup>80</sup>

Both **81** and **82** are common semiochemicals released by flowers or leaves, e.g. in response to insect feeding.<sup>82</sup> In combination with other floral volatiles and visual signals they elicit feeding responses in *Heliconius* butterflies.<sup>83</sup> The attractive or repellent activity of linalool (**81**) seems to be context-dependent, as similarly found for other signalling compounds

in this genus.<sup>72,80</sup> A terpene synthase producing **81** was characterized in another species, *H. melpomene*, although this species does not show a pupal-mating trait.<sup>84</sup>

The courtship behaviour of *Heliconius* butterflies comprise complex and diverse behavioural acts involving the use of multiple signals at different phases in mate recognition and choice. Males are first attracted by colour wing pattern serving as a mating cue; in short-range other signals such as pheromones are likely to mediate recognition and mating.<sup>76,85–88</sup> In species involved in mimicry complexes, recognition is likely primarily based on pheromones in co-mimics, where visual signals alone are not sufficient to identify conspecifics.<sup>86,89–92</sup> Sympatric species with the most similar colour pattern also seem to have developed the most divergent and conspicuous courtship rituals.<sup>76</sup> It is likely that the CSG and androconial extracts, and their specific composition, play different roles at different stages of courtship and may influence reproductive success and isolation between species.<sup>75,88</sup> In contrast to the general pattern observed in Lepidoptera, the chemical signature between *Heliconius* species, even sister pairs, is often different. Nevertheless, while mimetic pairs contain different scent bouquets, they also have some compounds in common, whereas sympatric species, mimetic and non-mimetic, have more distinct compound compositions.<sup>88,93,94</sup>

The use of AAs in *Heliconius* was initially postulated by Gilbert<sup>95</sup> and except in the Pieridae and the moth *Heliothis virescens*,<sup>96</sup> no other use of AA in Lepidoptera has been reported.<sup>76,83,97–99</sup> While AA are found in a wide range of insects, chemical structures have only been identified for a few species.<sup>72,96</sup> In *H. melpomene*, behavioural test showed (*E*)- $\beta$ -ocimene (**85**) to act as an AA, effectively repelling males when applied to unmated females.<sup>72,78</sup> A reliable method for the synthesis of pure (*E*)- $\beta$ -ocimene (**85**) has been developed using a Wittig-reaction as the key step.<sup>100</sup> The CSG of *H. melpomene* also contains a less volatile matrix of C<sub>16</sub>–C<sub>18</sub> fatty acid esters of ethanol, 2-propanol, 1-butanol, isobutanol, 1-hexanol and (*Z*)-3-hexenol that moderates the evaporation rate of **85**.<sup>72</sup> By contrast, freshly emerged males and females lacked volatile compounds, except for guaiacol (**84**), and only C<sub>16</sub>- and C<sub>18</sub>-fatty acids together with cuticular compounds such as hydrocarbons, cholesterol, and squalene. Not until five days after eclosion, had males developed the whole bouquet of compounds.<sup>72</sup> Because male *Heliconius* do not become sexually active until several days after eclosion, the lack of these compounds in females and younger males supports a role in mating behaviour.<sup>75</sup>

The AA pheromones vary both qualitatively and quantitatively between *Heliconius* species. (*E*)- $\beta$ -Ocimene (**85**) can also be found in distantly related *Heliconius* species. In *H. erato* **85** is found in the CSG at around one-tenth the amount of *H. melpomene*, while it is absent in *H. cydno*, a closely related species to *H. melpomene*.<sup>72,78,90,101</sup> It is a very common plant volatile, which is found in large amounts in flowers on which adult *H. melpomene* feed, and elicits a strong electroantennographic antennal response in both males and females. As discussed for **81**, **85** seems to have two context-dependent functions, the attraction to plants and the repulsion from mated females.<sup>72,102</sup> While an additional uptake of **85** through

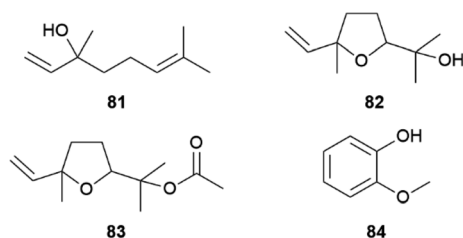


Fig. 15 Volatile compounds of pupae (**81**–**83**) and freshly emerged *Heliconius* (**84**).



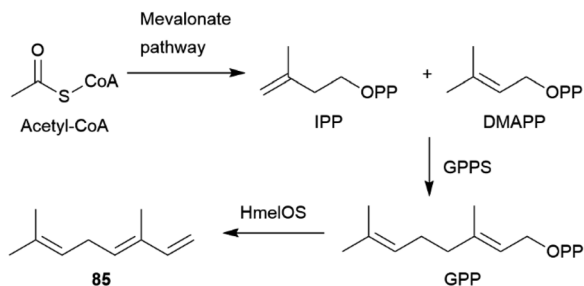


Fig. 16 Biosynthesis of (*E*)-β-ocimene (**85**) in *Heliconius* based on Darragh *et al.*<sup>84</sup>

feeding cannot be ruled out, feeding experiments with freshly eclosed males with isotopically labelled D-<sup>13</sup>C<sub>6</sub>-glucose have shown that *H. melpomene* is able to synthesize **85** *de novo*, and that it is transferred onto females during mating. Glucose can be converted into doubly labelled acetate *via* the citric acid cycle and enters the mevalonate pathway of terpene synthesis.<sup>72</sup> Combining gene identification through QTL (quantitative trait locus) mapping, heterologous gene expression and functional analyses, Darragh *et al.*<sup>84</sup> were able to identify the monoterpene synthase HmelOS which catalyses the conversion of geranyl pyrophosphate (GPP) to **85** in *H. melpomene* (Fig. 16). HmelOS is unrelated to previously described plant and insect terpene synthases and is the first described **85** synthase in animals. Although the sister species *H. cydno* possess an orthologous enzyme HcydOS, no evidence for its activity as a terpene synthase was found, leading to absence of **85** in this species.<sup>84</sup> Other AAs are hexyl 3-methylbutanoate (**89**) in *H. cydno* and benzyl salicylate (**86**) in *H. charithonia* (Fig. 17).<sup>72,78,103</sup>

In general, the composition of CSG in *Heliconius* varies considerably among species, in composition and number of compounds, comprising species-specific compound mixtures. These mixtures can include esters, lactones, terpenes, alcohols, ketones, aromatic compounds and can contain up to 350 components.<sup>70,72,73,78,87,90,94,104</sup> For both androconial and CSG chemical profiles most variation is explained by species identity rather than geographic location, which could be important for chemical mimicry and species recognition.<sup>90</sup> Estrada *et al.*<sup>78</sup>

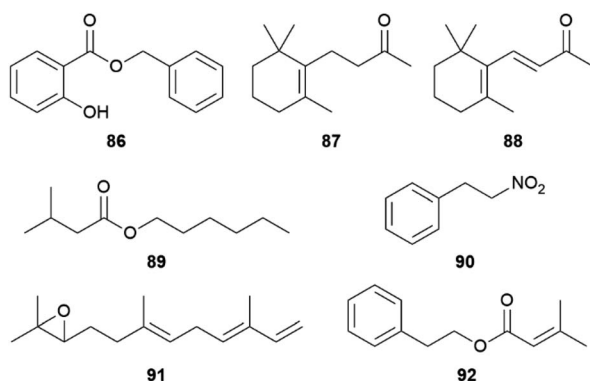


Fig. 17 Antiaphrodisiacs in *Heliconius* (**86**, **89**) and major compounds of the CSG.

have shown that pairs of species belonging to the pupal mating clade have a more similar pheromone composition to each other than pairs of species in the non-pupal mating clade, where the chemical composition has diverged faster under the influence of sexual selection. This is also observable for the major volatile compounds of the CGS of different *Heliconius* species.

The AA compositions of the non-pupal mating clade diverged between sister species and closely related species, whereas in the pupal-mating clade the major compounds appear to be conserved across more divergent species. In *H. charithonia*, **86** is the single major volatile compound while in *H. sara*, **86** is dominant together with (*Z*)-3-hexenyl decadienoate, with unknown locations of the double bonds. Also *H. hewitsoni* and *H. sapho* share the presence of **86** together with dihydro-β-ionone (**87**) albeit in *H. sapho* (*Z*)-3-hexenyl decadienoate is also a major compound. (*E*)-β-Ocimene (**85**) is shared between the pupal mater *H. erato*, where also a whole range of methyl, ethyl, hexyl, (*Z*)-3-hexenyl and other esters of dihydrofarnesoic acid are present,<sup>73</sup> and the non-pupal mater *H. melpomene*, *H. numata* and *H. hecale*. In *H. ismenius* (*E*)-α-ionone (**88**) is the major volatile and *H. cydno* and *H. pachinus* share the presence of hexyl 3-methylbutanoate (**89**).<sup>78</sup>

Although compounds usually occur in several species, compounds which are strong indicators of species identity can be found, as 3-oxooctyl and (*Z*)-3-hexenyl (*S,E*)-2,3-dihydrofarnesoate (**94** and **95**)<sup>73</sup> for *H. erato*, high concentrations of **85** for *H. melpomene* or eicosenol in *H. elevatus*. But also the occurrence of more than one compound can be specific for a species, *e.g.* in *H. timareta* the presence of butyl oleate (**96**) and (*Z*)-9-octadecen-13-olide (**105**) or (*Z*)-3-hexenyl isobutyrate (**97**) together with an unknown compound in *H. sapho* (Fig. 18).<sup>90</sup>

Dihydrofarnesoates have been synthesized through esterification of the respective alcohol with (*S,E*)-2,3-dihydrofarnesoic acid, obtained from (*2E,6E*)-farnesol (**29**) by enantioselective hydrogenation using Noyori's method, followed by Parikh-Döring and Pinnick-Lindgren oxidations.<sup>73</sup>

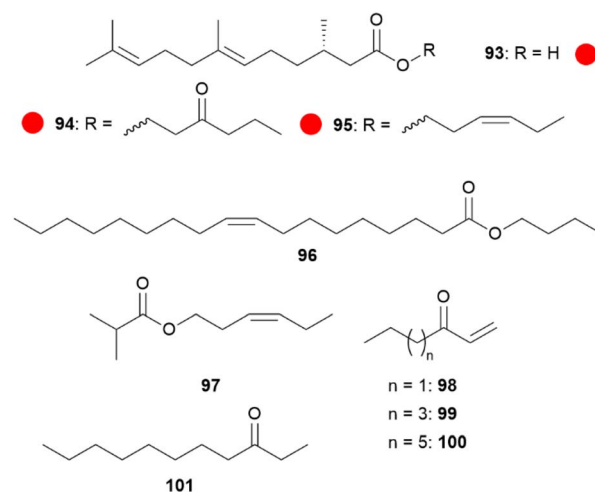


Fig. 18 Species-specific CSG compounds in *Heliconius*. The colour code indicates occurrence only in a single genus (•).



An interesting compound class present in some CSGs of *Heliconius* species are macrolides (Fig. 19). Phyllisolide (**102**) was the first macrolide pheromone identified in *Heliconius* with an AA function. Even with its very low concentration in the CSG of *H. erato phyllis*, it still elicits the strongest GC-EAD response next to the other active compounds 1-hexen-3-one (**98**), 1-octen-3-one (**99**), **85**, 3-undecanone (**101**) and (*E*)-2,3-dihydrofarnesoic acid (**93**) in the male antenna. This derivative of geranylacetone (**173**) is biochemically related to ferrulactone (**152**) and brassicalactone (**153**), which are pheromones of the Pieridae family (see below) and also degraded terpenoids.<sup>105,106</sup> Synthetically, **102** has been obtained from (*E*)-geranylacetone (**173**) with a Riley oxidation followed by an enantio- and stereoselective hydrogenation, and subsequent Jones oxidation and esterification from the corresponding acetal. A reduction to the respective alcohol and enantioselective enzymatic acetylation followed by basic hydrolysis and final macrolactonization delivered the natural stereoisomer **102**.<sup>105</sup> Another species where macrolides are abundant is *H. pachinus* and its very close relative *H. cydno*, which are inter-fertile.

In both sexes fatty acid derived macrolides can be found in the CSGs. The macrolides present are the 14-membered lactones (9*Z*,11*E*,15*Z*)-octadeca-9,11,15-trien-13-olide (**103**), (*S*)-coriolide (**104**), (9*Z*,13*S*)-octadec-9-en-13-olide (**105**) and the 12-membered lactone (9*Z*,11*S*)-octadec-9-en-11-olide (**106**). Considering the markedly lower concentrations in females and absence in younger male adults and pupae, these lactones might be similarly used as AAs, but there is no evidence yet.<sup>70,104</sup> The macrolide **104**, which was previously found in the seed oil of *Monnina emarginata*<sup>107</sup> and the fungus *Stagonospora*,<sup>108</sup> had been reported before<sup>104</sup> for male *H. pachinus* as (*R*)-**104**. Synthetically, **104** has been obtained through enzymatic oxidation of linoleic acid, followed by a reduction and final macrolactonisation.<sup>109</sup> These lactones are likely formed by

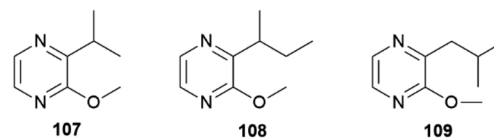


Fig. 20 Pyrazines in *Heliconius*.

lipoxygenases acting at linolenic, linoleic and oleic acids, followed by macrolactonization.<sup>70</sup> In addition to courtship signals, warning odours can also be found in the CSG of *Heliconius*, namely pyrazines. Pyrazines act as general warning odours of chemically defended insects and are known to deter rats and birds.<sup>110,111</sup> In *H. melpomene* 3-isopropyl-2-methoxypyrazine (**107**), 3-sec-butyl-2-methoxypyrazine (**108**) and 3-isobutyl-2-methoxypyrazine (**109**) are present,<sup>72</sup> while in the subspecies *H. m. malleti* only **107** can be found (Fig. 20). The co-mimic species *H. timareta florenzia* contains **108**.<sup>88</sup> Pyrazine **109** is also found in *Actinote pelleria* of the tribe Acraeini of the Heliconiinae.<sup>111</sup>

A thorough analysis of the CSG contents of 115 individuals of *H. erato* belonging to different mimicry groups revealed that they show a mimicry group specific distribution of volatiles and also other compounds.<sup>73</sup> Therefore, mimicry groups cannot only be assigned due to wing patterns, but also due to the composition of the CSGs. The most abundant volatiles within the CSG were **101** and 3-nonanone, **85**, the vinyl ketones **98**, **99**, and **100**, 2-nitroethylbenzene (**90**), **31**, **89**, benzyl cyanide (**162**), and some other compounds including 2-phenylethyl 3-methyl-2-butenate (**92**).<sup>73</sup> The CSG compounds are generally recruited *via* different biosynthetic pathways, such as terpene and fatty acid pathways, but include also derivatives of primary metabolites or apocarotenoids, and aromatics. A key enzyme activity seems to be ester ligation, because esters in open or cyclic form are a dominant class of CSG compounds of the Heliconiinae.

*Dione vanillae* (formerly *Agraulis vanillae*),<sup>112</sup> belongs to one of the most primitive genus of the Heliconiini.<sup>113</sup> Both sexes possess abdominal scent glands which are quickly everted when a butterfly is captured or disturbed. The abdominal gland composition is identical in males and females, with sulcatone (**110**) as the major volatile (Fig. 21). Sulcatone (**110**) is known as

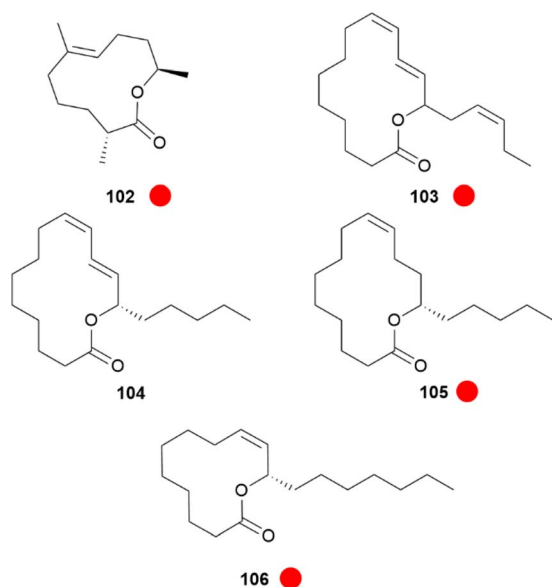


Fig. 19 Macrolides present in the CSG of *Heliconius*. The colour code indicates occurrence only in a single genus (●).

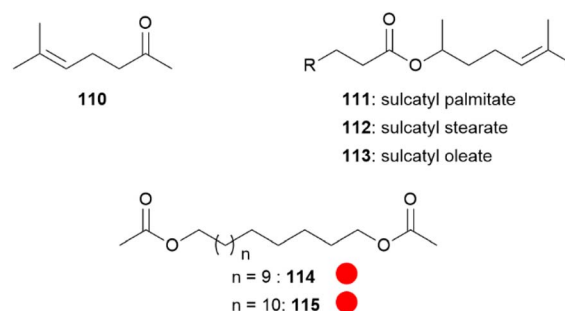


Fig. 21 Compounds present in *Dione vanillae*. The colour code indicates occurrence only in a single genus (●).





a defensive allomone of ants and cockroaches, widespread in nature, and is rejected by birds.<sup>113,114</sup> Other constituents of the scent bouquet are the sulcatyl fatty acid esters **111–113**, 1,15-pentadecanediyl (**114**) and 1,16-hexadecanediyl (**115**) diacetates.<sup>113</sup> As adults of this species are designated as prey items by invertebrate and vertebrate predators, the bouquet seem to exhibit a protective function,<sup>113</sup> especially the most volatile compound **110**.

## 4.2 Androconia

Different behavioural assays have demonstrated the importance of androconial chemical signals in *Heliconius* mating behaviour.<sup>75,85,87–89</sup> The hindwing androconia emit lower amounts of less complex bouquets that is usually chemically different from that of the CSG. The compound bouquets are species specific<sup>94</sup> and influences mating behaviour.<sup>75,87</sup> The majority of these compounds are fatty acid-derived compounds including aldehydes, alcohols, acetates or esters preferentially with a C<sub>18</sub> and C<sub>20</sub> chain, together with some alkanes.<sup>94</sup> One third are considered to originate from the phenylpropanoid pathway, active in plants, taking up during feeding.<sup>77,115</sup> All double bonds of fatty acid derivatives in *Heliconius* are located at the ω-9 position, pointing to an active Δ9-desaturase, contrasting to the Δ11-desaturase present in *Bicyclus* (see below).<sup>68,94</sup> The high chemical diversity of the bouquet can be achieved by activating or deactivating enzymes/genes responsible for certain biosynthetic transformations, as a general model for the biosynthesis proposed (Fig. 22).<sup>116</sup> For instance in *H. melpomene* an alcohol oxidase acting on saturated alcohols furnishing aldehydes seems to be active, which is inactive in *H.*

*cydno*.<sup>94</sup> Subtle changes in a few enzyme activities therefore might result in different scent gland mixtures, leading to differences between species, subspecies or even individuals.<sup>94</sup>

As with the CSG, in most species there are single androconial compounds that are strong indicators for species identity (Fig. 23). For *H. eleuchia* it is hexahydrofarnesyl acetone (**117**), homovanillyl alcohol (**118**) in *H. elevatus*, octadecanal (**116**) for *H. melpomene*, methyl 4-hydroxy-3-methoxybenzoate (**120**) in *H. sapho*, and 5-decanolide (**121**) for *H. timareta*.<sup>90</sup>

Octadecanal (**116**), which is mostly absent in other *Heliconius* species,<sup>87,117</sup> serves as a volatile courtship signal. It is a common pheromone gland component and has been tested in eight species of Lepidoptera across a variety of families including *Heliconius*, but not all showed activity.<sup>88,106,117,118</sup> Electrophysiology and behavioural experiments demonstrated **116** as a biologically active pheromone component involved in mating decisions in *H. melpomene* and, surprisingly also in *H. cydno*, although it is not produced by the latter species.<sup>77,117</sup>

In species recognition trials females discriminated against conspecific males with blocked pheromone transmission, suggesting that females are actively involved in mating decisions.<sup>75</sup> This would be in line with the species-specific androconial secretion.<sup>87,90,94</sup> Likewise *H. erato* can distinguish their own wings from those of their *H. melpomene* mimic, but not when the wings were washed with hexane.<sup>85</sup> Also the co-mimic species *H. melpomene* and *H. timareta florenci* in Colombia display strong reproductive isolation. The bouquet of *H. t. florenci* consisting of heneicosane, syringaldehyde and limonene (**150**) contrasts with those of *H. melpomene* dominated by **116**.<sup>88</sup> Even in species pairs that differ in their color pattern, the total level of premating sexual isolation perceived in experimental conditions are higher (97%) than isolation considering male-driven color choice only (75%),<sup>119</sup> implying contribution of nonvisual signals, like chemical scents to species specific mate selection.<sup>87,91</sup>

A survey of 33 of the 69 species of the Heliconiini confirmed species-specific androconial bouquets, especially in *Heliconius*, indicating a role in reproductive isolation within this tribe. The fatty acid metabolic pathway was recruited by more advanced

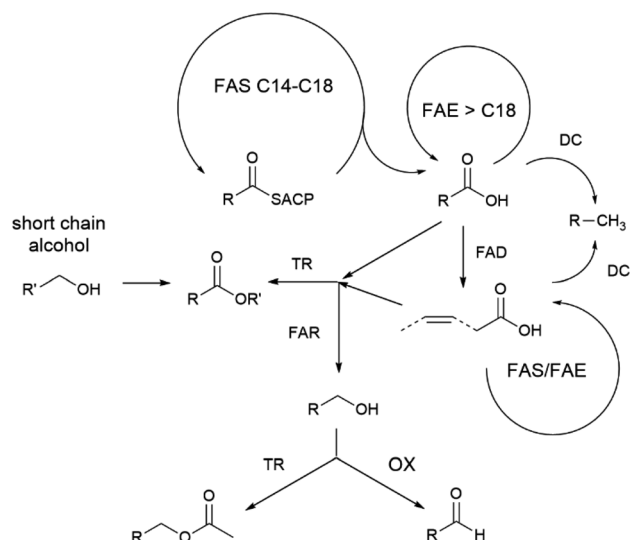


Fig. 22 Proposed biosynthetic pathways to different fatty acid metabolites used by *Heliconius* adapted from Mann *et al.*<sup>94</sup> FAS fatty acid synthase; DC decarboxylase; FAD fatty acid desaturase; TR transferase; FAR fatty acid reductase; FAE fatty acid elongase; OC oxidase; ACP acyl carrier protein. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Journal of Chemical Ecology*, The scent chemistry of *Heliconius* Wing Androconia, F. Mann *et al.*, Copyright (2017).<sup>94</sup>

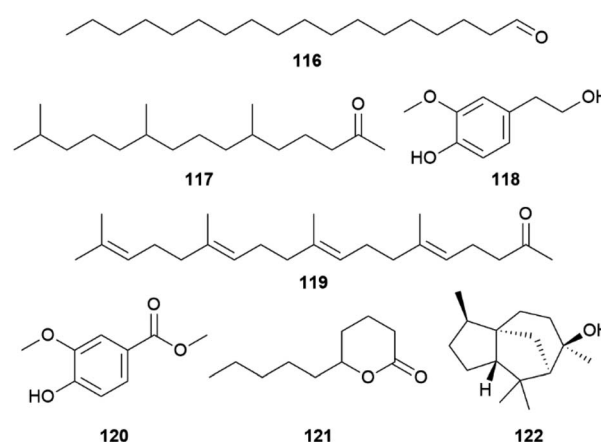


Fig. 23 Species indicator androconial compounds in *Heliconius*.

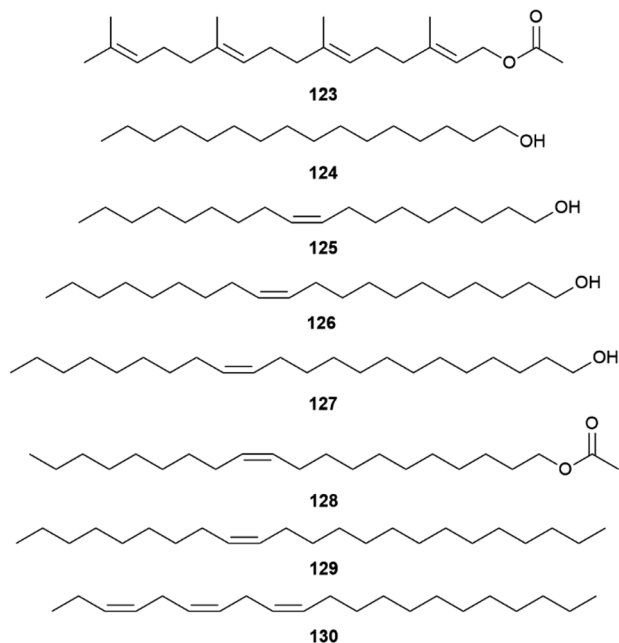


Fig. 24 Fatty acid metabolites of androconia of various Heliconiini and geranylgeranyl acetate (123).

genera to arrive at more complex blends, while basal lineages are dependent on plant metabolites such as **117–120**, or geranylgeranyl acetate (**123**) of *H. erato* and *H. himera*.<sup>120</sup> The fatty acid derived compounds include mostly compounds similar to type I moth pheromones compounds such as **116** and hexadecanal (**132**) of *H. aoede*, 1-hexadecanol (**124**), (Z)-9-octadecen-1-ol (**125**) of *H. xanthocles*, (Z)-11-eicosen-1-ol (**126**) of *H. atthis*, (Z)-13-docosen-1-ol (**127**) of *Eueides tales*, (Z)-11-eicosenyl acetate (**128**) of *E. aliphera*, but also alkanes such as heneicosane, tricosane, and (Z)-9-tricosene (**129**) of *H. charithonia*, as well as (3Z,6Z,9Z)-3,6,9-henicosatriene (**130**) of *H. demeter*, a typical type II moth pheromone (Fig. 24).<sup>120</sup> *H. erato* is an exception, lacking any fatty acid metabolites. The androconia contain small amounts of geranylgeranylacetone (**119**),<sup>94</sup> but the major component is terminally oxidized **119** conjugated to fatty acids, which is non-volatile.

The Acraeini are another tribe of the subfamily Heliconiinae, which is highly paraphyletic, involving the genus *Cethosia*, the lacewing butterflies. In the dimorphic species *Cethosia cyane cyane* males actively chase females during courtship using only visual signals to distinguish gender, which is possible due to their sex-specific wing colouring.<sup>121</sup> Nevertheless, behavioural assays showed that olfactory signals also assist at close range for accurate identification. SPME analysis of living males and females showed little differences in produced compounds, only cedrol (**122**) was exclusively detected in females (Fig. 23). Despite the fact that heneicosane and linalool oxide (**82**) also show EAG activity, only responses to **122** were significantly different between females and males. Behavioural assays proved the participation of **122** in mate recognition of females at close range.<sup>121</sup>

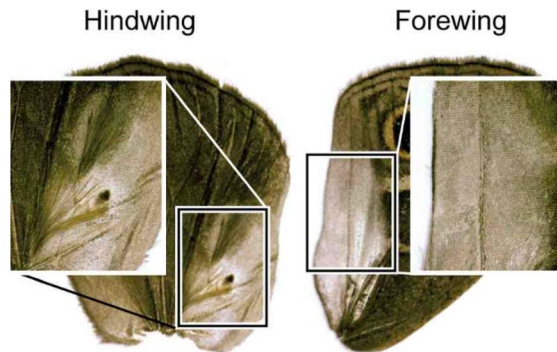


Fig. 25 Wing androconia of *Bicyclus anynana* with patch of hairs.<sup>131</sup> The picture is reproduced from *Plos One*, C. M. Nieberding *et al.*, under an unrestricted CC licence.

## 5 Satyrinae

The Satyrinae, the browns, are a subfamily of the Nymphalidae. These butterflies generally consume plants lacking secondary compounds and due to this they are mostly palatable, and lack the typical aposematism of other butterflies.<sup>122</sup> *Bicyclus*, an African genus from the tribe Elymnini, is the best studied butterfly genus with regard to sexual, including olfactory, communication.<sup>123–127</sup> *Bicyclus* represents a clade where multiple species live mostly in sympatry and display a diversity of visual signals.<sup>128</sup> Males of this genus are primarily differentiated on the basis of the location and number of male scent glands (androconia) and modified hair-like scales covering these glands, present at the hind- and forewings (Fig. 25).<sup>129,130</sup> The modified hair-like scales are presumably assisting in spreading male scent and therefore are defining a diversification of the androconia, which might indicate the importance of scent during speciation and divergence within the genus.<sup>127</sup> The use of species-specific male pheromones have been proven.<sup>130</sup> The chemicals emitted by male androconia, received in the primary sensory organs on the antennae, are important in female choice and are involved in male–male recognition.<sup>127</sup>

Pheromone transfer likely happens during flickering and thrust, where the androconial hairs fan out and become clearly visible.<sup>129,131</sup> The existence of AA has not been reported and is

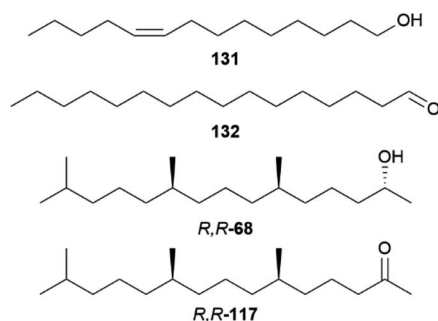


Fig. 26 Male androconial sex pheromone blend of *Bicyclus anynana* (**131**, **132**, *R,R*-**68**) and ketone *R,R*-**117**.



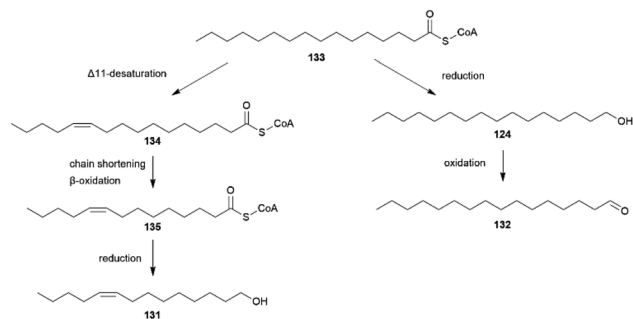


Fig. 27 Biosynthesis of fatty acid derived pheromones **131** and **132**, adapted from Liénard *et al.*<sup>68</sup>

not suggested based on butterfly behaviour.<sup>123</sup> Chemical and visual signals seem to be equally important in female mate choice, and are most likely involved in mate quality assessment.<sup>127,132,133</sup>

*Bicyclus anynana* produces a close-range male sex pheromone (MSP) blend in the androconia consisting of the GC-EAD active and sex-specific compounds (*Z*)-9-tetradecenol (**131**), hexadecanal (**132**) and (2*R*,6*R*,10*R*)-2,6,10-trimethylpentadecan-2-ol (*R,R,R*-**68**, Fig. 26). The biosynthesis of these compounds begins after adult emergence and is associated with the androconia. While **131** and **68** were found to be mainly produced in the forewing androconia, **132** is only produced in the hindwing together with minor amounts of **68**, whereas the hair-like scales are not involved in pheromone production.<sup>131</sup>

Hedenström *et al.* were able to verify through derivatization methods that the stereochemistry of **68** and the corresponding ketone **117** is always (2*R*,6*R*,10*R*)-**68** and (6*R*,10*R*)-**117** for different *Bicyclus* species.<sup>125</sup> This indicates that **68** and **117** are related to substances found in the butterfly or larval diet, determining the stereochemistry. The biosynthesis of **117** has been investigated in the pierid butterfly *Pieris brassicae*, showing plant-derived (*E*)-phytol (**154**) with a natural all-*R*-configuration as a precursor (see below).<sup>125,134</sup> Through implementation of an asymmetric synthetic strategy, **68** and **117** can be obtained stereoselectively starting from sulcatone (**110**) and citronellal acid with a lipase-catalyzed resolution as a key step.<sup>125</sup> Alcohol **68** is a well-known sex pheromone of pest insects, such as the rice moth, *Corcyra cephalonica*, the African sugar-cane borer moth, *Eldana saccharina*, and the oil palm bunch moth, *Tirathaba mundella*, highlighting the widespread occurrence of this compound among Lepidoptera.<sup>135</sup> On the other hand, **131** and **132** display type I moth pheromone structures.<sup>1,131</sup> Liénard *et al.* showed that **131** and **132** are produced *de novo* in *B. anynana* via a biosynthetic pathway shared between females of many moth species, providing the first evidence of conservation and sharing of ancestral genetic modules for the production of fatty acid-derived pheromones over a long evolutionary timeframe.<sup>68</sup> Both compounds derive from palmitic acid, a known essential precursor for female moth pheromone biosynthesis.<sup>68,136</sup> For the production of **131** palmitic acid firstly undergoes a desaturation towards (*Z*)-11-hexadecenoic acid involving a fatty acyl  $\Delta$ 11-desaturase,

followed by one cycle of chain shortening *via*  $\beta$ -oxidation to (*Z*)-9-tetradecenoic acid, and finalized through a reduction to the corresponding alcohol **131** (Fig. 27).<sup>68</sup> Aldehyde **132** is synthesized from palmitic acid in two steps over a reduction to **124**, followed by oxidation to the aldehyde.<sup>68</sup>

Among all compounds of the scent organs identified in *Bicyclus*, saturated and unsaturated fatty alcohols, aldehydes, esters and hydrocarbons account for almost two thirds, suggesting a close biosynthetic relationship.<sup>130</sup> The MSP composition was found to be a reliable indicator of male identity, level of inbreeding, as well as age.<sup>132,133</sup> MSP ratios also vary in individuals and could contain information on male individuality and provide the opportunity for interindividual recognition.<sup>132</sup> Headspace analyses showed that males can actively control MSP emission and adjust to an increased male–male competition.<sup>137</sup> Other effects such as phenotypic plasticity in MSP production,<sup>124</sup> geographical variation, likely driving reproductive isolation and speciation,<sup>138</sup> reproductive character displacement MSP composition of different *Bicyclus* species,<sup>123</sup> learning of visual signals in mate choice,<sup>139</sup> and plastic female preferences for a male pheromone odour blend influenced by early pheromone odour experiences<sup>140</sup> have been studied.

A more precise function of the MSP compounds **131**, **132** and **68**, as well as their relative importance in *B. anynana* are disputed as laboratory experiments are not always comparable to natural conditions.<sup>141</sup> A comparison of wing extraction and headspace sampling methods underlined the importance and differences of sampling methods.<sup>137</sup> While wing extracts only reflect stored compounds, it does not necessarily represent the compounds actually emitted during courtship. Whereas headspace analysis maybe better at representing the released scent, although valid for a confined space, it is not known if it is comparable to what a female receives.<sup>137</sup> Nevertheless, EAG recordings at least underline the possibility of the detection of released compounds. The MSP ratios obtained for *B. anynana* with both methods differed significantly.<sup>137</sup> Although this discussion was centred on the results obtained from *B. anynana*, it is true for all kinds of analysis of volatile, behaviour-mediating compounds.

In *B. martius sanaos* some novel wing-specific esters were identified, not present in females, representing possible pheromone candidates.<sup>130</sup> In male wing extracts ethyl, isobutyl and 2-phenylethyl hexadecanoates (**136a–c**) and (*Z*)-11-

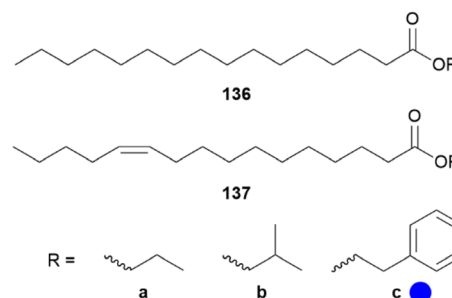


Fig. 28 Wing-specific ester of androconia of *Bicyclus martius sanaos*. The colour code indicates occurrence only in a single family (•).





hexadecenoates (**137a–c**) as well as ethyl benzoate, 2-phenyl-ethyl tetradecanoate and 2-phenylethyl octadecanoate were found, some already known from *Heliconius* (Fig. 28).<sup>70,72,130</sup> Wang *et al.* showed that incorporation of D-labelled amino and fatty acids into the associated esters only take place in patches of the forewing, indicating that only the forewings contain active biosynthetic enzymes responsible for ester synthesis.<sup>130</sup>

The suggested biosynthesis proposed that the required alcohols can be synthesized *de novo* starting from L-alanine, L-valine and L-phenylalanine after a sequential process of transamination, decarboxylation, and reduction. An active  $\Delta 11$ -desaturase, previously reported in *B. anynana*, transforms hexadecanoic acid into hexadecenoic acid, both acids being then enzymatically esterified with the respective alcohols.<sup>68,130</sup> This biosynthetic route seems to be of widespread use within the butterflies, found also in Heliconiini, Ithomiini, or pierids (see Fig. 22).

During courtship non-random gustatory contacts also occur between specific male and female body parts.<sup>142</sup> As described for *Pieris rapae*, alkanes and methyl-branched alkanes build the majority of cuticular compounds together with aldehydes, alcohols, ketones and acids.<sup>142,143</sup> As the composition is associated with body parts, some compounds may potentially provide additional fine-scaled information, complementing already furnished information by male sex pheromones.<sup>142</sup>

A different species of Elymniini, *Elymnias thryallis*, found in Papua New Guinea, possess a scent gland at the end of the abdomen which secretes a fluid when disturbed, maybe acting as a deterrent against predators or as a courtship pheromone. The main compound of the secretion was found to be the monoterpene elymniafuran (**138**) as well as trace dehydrated compounds *i.e.* (*E*)- and (*Z*)-4-methyl-2-(2-methyl-1,3-butadienyl)furan (**139**), which are not found in other natural sources (Fig. 29).<sup>144</sup> Elymniafuran (**138**) has been synthesized using an enantioselective vinyl-Grignard addition under Taddol catalysis as a key step.<sup>144</sup>

In the subtribe *Lethina*, males of the species *Lethe marginalis* possess a faintly sweet wing scent, whereas females are scentless. The male scent secretion consisted mainly of  $\gamma$ -delta-lactone (**63**) and some tridecane, without a known function.<sup>145</sup> With 80 ng present in the forewing and 73 ng in the hindwing, **63** was only found in small quantities compared to scent substances of some other butterflies.<sup>145,146</sup>

The use of an electronic nose in the investigation of volatile organic compounds (VOCs) during courtship behaviour of two *Hipparchia* species indicated a role for chemical signals in mate and species recognition during early stages of interaction.<sup>147</sup> While the two sexes of *H. fagi* displayed a similar VOCs pattern,

it is significantly diverged in *H. hermione*. VOCs patterns between females of the two species were different, but those of males were not. The structures of the compounds responsible for this variation have not been evaluated so far.<sup>147</sup>

## 6 Pieridae

The Pieridae are a family of medium-sized butterflies, comprising the subfamilies Dismorphiinae, Pierinae, Coliadinae and Pseudopontiinae. This family is monophyletic and mating plugs are found in all subfamilies except Dismorphiinae.<sup>6,148</sup> Most males display male-specific androconia. These scent gland patches are oval and flaky and arranged in dense clusters of scales concentrated in the area where the fore- and the hindwings overlap, without protection to prevent evaporation except overlapping of wings when at rest.<sup>149–151</sup> While Dismorphiinae and Coliadinae predominantly feed on Fabaceae, Pierinae were able to specialize on Brassicaceae due to the evolution of a nitrile-specifier protein (NSP) that detoxifies prevalent glucosinolate defences and allowing the evolution of a much more diverse subfamily than its sister clades.<sup>152</sup>

### 6.1 Dismorphiinae

*Leptidea*, a cryptic complex of butterflies, is a genus of the mimic sulphurs and includes several palearctic species.<sup>153,154</sup> The sister species *Leptidea sinapis* and *Leptidea reali* can only be distinguished using genital preparation or DNA sequencing but females nonetheless only accept conspecific males.<sup>155</sup> During their courtship males sit opposite to the females while oscillating their proboscis in front of them. Females can signal mating acceptance by making their abdomen accessible for male copulation attempts, thereby likely disseminating an aphrodisiac pheromone. Males will give up after some time,<sup>153,155,156</sup> leading to a quite unique long-lasting courtship, which can take up to 11 min until females accept mating, implying a longer recognition time of conspecifics.<sup>155</sup>

SPME analysis of both sexes of *L. reali* and *L. sinapis* support the use of species-specific volatiles in species recognition.<sup>155</sup> While both sexes emit methyl salicylate (**48**), limonene (**150**) and 1-octanol, (*E*)- $\beta$ -ocimene (**85**) seems to be a female specific volatile. Males additionally emit methyl benzoate (**141**), detected in 90% of *L. reali* and 30% of *L. sinapis* individuals. In about half of *L. sinapis* both sexes also emit dihydroisophorone (**142**) and cyclonol (**143**, Fig. 30).<sup>155</sup> Only when the additional volatiles **142** and **143** are emitted by female *L. sinapis*, *L. reali* males can distinguish between the species and abort courtship, while the absence is leading to a longer giving-up time. The additional volatiles **142** and **143** facilitate the mating decision for females

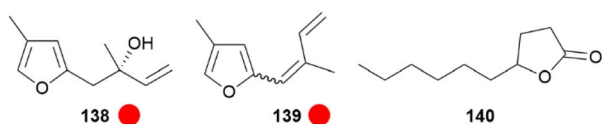


Fig. 29 Scent constituents of *Elymnias thryallis* and *Lethe marginalis*. The colour code indicates occurrence only in a single genus (•).

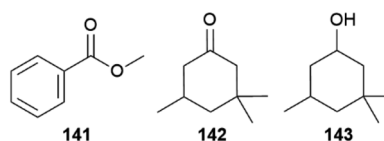


Fig. 30 Volatiles found in *Leptidea* involved in species recognition.



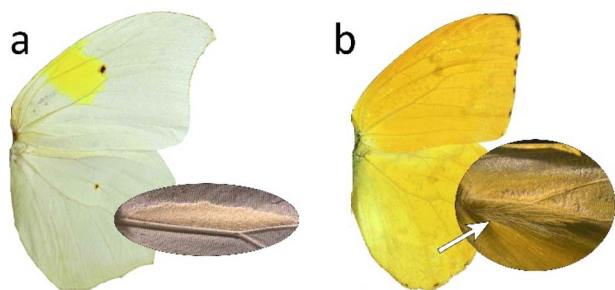


Fig. 31 Male dorsal wing color patterns of *Colias*-clade butterflies with androconia details (circles to the right) of (a) *Anteos clorinde* and (b) *Phoebis argante*. The white arrow indicates a tuft of hair-like scales.<sup>150</sup> Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Organisms Diversity & Evolution, Specialized androconial scales conceal species-specific semiochemicals of sympatric sulphur butterflies (Lepidoptera: Pieridae: Coliadinae), C. E. B. Nobre *et al.*, Copyright (2021).<sup>150</sup>

of *L. sinapis*, together with species-specific wing beats of males, while *L. reali* females must be sure about the absence of these volatiles and wing beats, explaining the higher variance in time to mate.<sup>155</sup>

## 6.2 Coliadinae

In the subfamily Coliadinae, the sulphurs, not all species feature androconia but often other secretory organs can be found, e.g. *Phoebis argante* males also display additional hair-pencils below the androconial patch (Fig. 31).<sup>150,157</sup> The first pheromones were described for the genus *Colias*.<sup>150,158,159</sup> In the secondary sympatric sister species *Colias philodice* and *Colias eurytheme*, male-specific secretory cells can be found on the hind wing<sup>158,160</sup> that have showed qualitative compositional differences.<sup>159</sup>

Male *C. philodice* produce the fatty acid hexyl esters **144–146**, representing their sex pheromone, while 13-methylheptacosane (**148**), specific for *C. eurytheme*, together with heptacosane and nonacosane, a sex-pheromone with a low-to-moderate aphrodisiac activity (Fig. 32).<sup>159,161</sup> In comparison, female wings of both species only feature straight-chain hydrocarbons, which are also present in males in comparable quantities.<sup>159</sup> Despite their low volatility **148** and the esters **144–146** exhibit significant

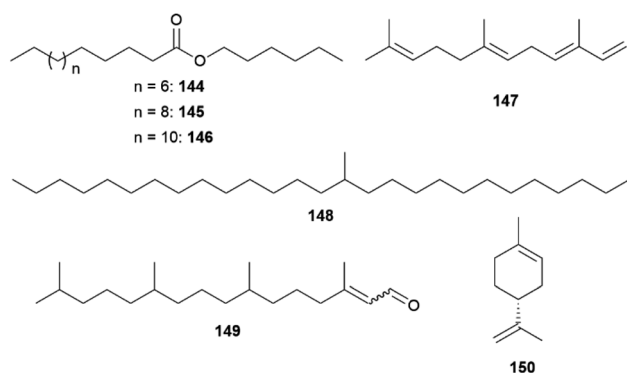


Fig. 32 Sex-pheromones of Coliadinae.

electrophysiological activity and display olfactory signals for mating.<sup>159</sup> While females of *C. philodice* rely solely on olfactory signals to select conspecific males,<sup>159</sup> the specific UV iridescence of male wings seem to be more important for mating success than pheromones in *C. eurytheme* and allows females to avoid heterospecific matings.<sup>162,163</sup>

In *C. eurytheme* pheromones also seem to be implicated in intra-specific mate choices since the UV reflectance and pheromone traits covary with age.<sup>162</sup> While hydrocarbons are commonly found in epicuticular wax layers of insects,<sup>161</sup> the amount of **148** varies greatly among individuals.<sup>162</sup> The individual blend of *C. eurytheme* males stabilize after 12 h post-eclosion and **148** is depleted over time, whereas heptacosane increases. In this case, the low volatility of components ensures the slow depletion over the lifetime of the male but requires very close range aerial detection by females or even contact.<sup>161</sup>

The distant clade *C. erate poliographus* also displays a sexual dimorphism in composition of epicuticular substances. The secretory organs, which are more abundant in the forewings, display a male-specific mixture, comprising of predominantly **145**, small amounts of **144** and **146**, octadecanal (**116**), and hexadecenoic and octadecanoic acids. Surprisingly, **148** was also found as a minor component in both sexes, and **145** and **116** were also present in females in lower concentrations.<sup>157</sup> Identical to *C. philodice*, *C. e. poliographus* seem to use hexyl esters as an aphrodisiac pheromone.<sup>157,159</sup> Age-dependent changes in the sex pheromone components indicating sexual maturation.<sup>132,157</sup> Octadecanal (**116**), **148** and hexyl esters **144–146** are increasing during days 1–7 after eclosion, whereas other linear alkanes have already reached a plateau at day 3.<sup>157</sup>

In other closely related non-mimetic Coliadini species-specific compositions of wing components were detected, suggesting use of intraspecific chemical communication.<sup>150</sup> The androconial blends of the co-occurring species *Anteos clorinde*, *A. menippe*, *Phoebis argante*, *P. philea*, *P. marcellina*, and *P. statira* revealed only a few dominant, autapomorphic compounds. Prevalent compounds across species are benzyl salicylate (**86**) in *A. menippe* and *P. marcellina* as well as a hexadecenoic acid with unknown double bond location in *P. statira* and *P. marcellina*. In general, higher similarities were found among males of *Anteos* compared to *Phoebis*, where only linoleic acid is shared between congeners.<sup>150</sup> The most characteristic compounds in the androconial blends were benzyl salicylate (**86**) and hexadecanoic and hexadecenoic acids in *P. marcellina*, 6,10,14-trimethylpentadecan-2-ol (**68**) in *P. argante*, limonene (**150**) in *P. marcellina*, as well as (*E,E*)- $\alpha$ -farnesene (**147**) in *P. marcellina* and *A. menippe*.<sup>150</sup>

Males of *Eurema mandarina*, the common grass yellow, of the tribe Euremini utilize male aphrodisiac sex pheromones released by androconia, inducing female acceptance.<sup>164</sup> Solvent extract of wings revealed hexahydrofarnesyl acetone (**117**) and (*E/Z*)-phytal (**149**, *E:Z* 4:1) as male specific compounds of the forewings (Fig. 32). The concentration of **117** is highest in the androconia where **149** is absent and is only detected in the anal cell, lacking androconia and intramembranous cells. While most other compounds are shared by both sexes, the oxygenated terpenoids **117** and **149** only occur in much smaller



amounts in females than in males.<sup>164</sup> Bioassays with virgin females demonstrated that **117** and **149** act synergistically as aphrodisiac sex pheromones, evoking mate acceptance in one-third of females. The response is weaker than with males, hinting at potentially other involved compounds. One compound alone elicits only little response in females.<sup>164</sup> As only responses were triggered when females were approached and touched by males, the signal acts in short-range or on contact.<sup>158,159,164</sup> As in *Colias*, the concentration of both **117** and **149** increases with age, implying there to be a representation of sexual maturity.<sup>161,164</sup>

### 6.3 Pierinae

In the subfamily Pierinae, the whites, adults commonly possess androconia, also called scent scales, on the dorsal wing surface, but the morphology can sometimes differ within genera and species.<sup>157,165</sup> The later stage of courtship, when females display a characteristic mate-refusal posture with spread wings and vertically lifted abdomen before eventually accepting mating, is assumed to be predominantly influenced by chemical signals.<sup>4,166,167</sup> Within this subfamily, in the tribe Pierini, males are known to use aphrodisiac pheromones and also to transmit antiaphrodisiacs with the spermatophore to females.<sup>97,99</sup> In *Pieris napi* males passively release citral (**151**), a 1 : 1 mixture of geranial and neral (Fig. 33). In EAG assays females were 10x more sensitive to **151** than males and the amount produced could influence mating success. Together with behavioural tests this proved that **151** acts as a courtship sex pheromone, necessary for successful courtship.<sup>168,169</sup> Furthermore, it allows males to assess the density of competing males in the vicinity.<sup>170</sup> Isomers of **151** are differently detected by glomeruli in the antennal lobe, but signals from both are needed for a behavioural response.<sup>171</sup>

Similarly, *P. rapae* and *P. brassicae* use aphrodisiac blends including two male specific macrolactones originating from their wing scent scales located over the entire wing area.<sup>106,172</sup> In

the small white *P. rapae* ferrulactone (**152**) was identified, previously known as a pheromone of the Rusty Grain beetle, *Cryptolestes ferrugineus*.<sup>173</sup> In the large white *P. brassicae* the related, but one isoprene unit bigger brassicalactone (**153**) is present. Both compounds reach a maximum concentration about ten days after emergence of adults.<sup>106</sup> Although GC-EAD experiments showed that **152** can be detected by both sexes of this species, **153** was only detected by female *P. brassicae*.<sup>106</sup> Other major components found in wing extracts are hexahydrofarnesyl acetone (**117**) in *P. rapae* and *P. brassicae*, and (*E*)-phytol (**154**), which is in *P. rapae* only a minor compound together with indole (**19**). The terpenoids **117** and **154** were also found in the respective female extracts but in lower amounts.<sup>100,106</sup> Mating-competition bioassays with an artificial aphrodisiac blend of **152** : **117** : **154** (21 : 12 : 1) for *P. rapae* and **153** : **117** : **154** (16 : 10 : 31) for *P. brassicae* showed an markedly enhancement in mating success for males with painted wings.<sup>106</sup> In contrast to *P. napi*, where the aphrodisiac compound is emitted more passively, in *P. rapae* *in vivo* monitoring using confined direct analysis in real time mass spectrometry (cDART-MS) of males indicated a rapid release during wing opening which seems to be triggered by a visual cue of a female together with other information, and only slightly passive emissions.<sup>174</sup>

In addition to these compounds, a plethora of about 100 components were detected on the wings, consisting predominantly of long-chain cuticular alkanes, secondary alcohols, diols and ketones, but also of more volatile compounds such as  $\beta$ -ionone, dihydroactinidiolide, decanal (**169**) or suspensolide (**155**), a isomer of **152** and a volatile of the Mexican fruit fly, *Anastrepha suspensa* (Fig. 33).<sup>175</sup> Electroantennographic experiments showed that not only the discussed pheromone components, but also other wing compounds are detected by the two species in a sex- and/or species specific manner. These include benzyl cyanide (**162**), tetradecanal, alcohol **68**, **149**, 1-hexadecanol, 1-octadecanol, as well as the hydrocarbons pentadecane, heneicosane, tricosane, 7-methyltricosane, tetracosane, pentacosane, heptacosane, nonacosane, triacontane as well as a mixture of mid-chain oxidized secondary alcohols, hepta- and octacosanols, as well as the terpenoid lactone 4,8,12,16-tetramethylheptadecan-4-olide (**156**).<sup>100</sup> These results and those discussed with *Colias* show that even compounds such as octacosanols or triacontanes can still be perceived as odours by the insects antennae, showing the ambiguity of the term “volatile”. Especially the hydrocarbons and terpenoids have been implicated as pheromones in other pierids. This indicates, that pheromonal communication in pierids is more intricate as described so far, although one does not have to forget that EAG only records an electric signal of the antennae, with no possible differentiation between simple odour receptors and those tuned for pheromone perception.

*P. rapae* additionally shows a chemical polymorphism of hydrocarbon profiles of the epicuticular wax layer of the antennae and other body parts, which seems to be of functional relevance.<sup>143</sup> Branched hydrocarbons that are predominantly found on antennae exhibit a lower melting point than linear ones, which are more dominant on the body, head and wings.

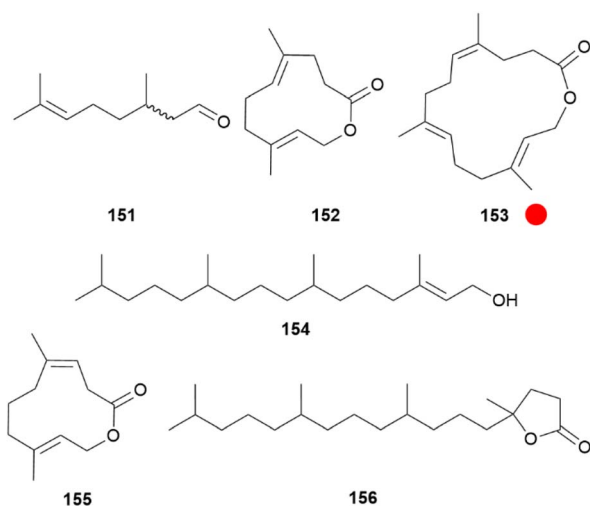
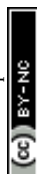


Fig. 33 Aphrodisiac compounds found in male *Pieris* species. The colour code indicates occurrence only in a single genus (•).





The antennae specific compounds could lead to higher water loss, higher fluidity and, maybe more relevant, to higher diffusion rates of compounds trapped from the gas phase.<sup>143</sup> Nevertheless, courtship involves antennal contact in many butterflies and specific antennal signals, maybe specific hydrocarbons, might be involved in ensuring courtship success.

The species specificity of the androconial secretions is underlined by the refusal of *P. melete* males by female *P. rapae*.<sup>176</sup> The allopatric subspecies *P. rapae rapae* and *P. rapae crucivora* display a strong premating isolation, although the pheromones **117** and **152** occur in both subspecies, albeit in different concentrations.<sup>151</sup> Females reject males of the other subspecies probably due to the diverging wing compound profiles, which are more different between males compared to females.<sup>151</sup> Even though no compounds are exclusively present in one subspecies or only in females, **152**, **162**, **117**, **154** and a phytol derivative are only present in males and appear in statistically higher amounts in *P. r. rapae*. However, males display identical courtship towards all females, despite females differing dramatically in wing color or UV reflectance, indicating a low stringency in the mate recognition system.<sup>151,177</sup>

Amongst males of *P. melete* and *P. napi japonica* morphological differences are slight and males of both species display very similar wing scent scales profiles containing undecane and the monoterpenes  $\alpha$ -pinene (**157**),  $\beta$ -pinene (**158**), myrcene (**159**), *p*-cymene (**160**), limonene (**150**), citral (**151**) (Fig. 34).<sup>178</sup> Species recognition was proposed to rely on the different E/Z ratios of **151** as well as the additional occurrence of linalool (**81**) in *P. n. japonica*.<sup>178</sup>

During mating, *P. napi* males transfer methyl salicylate (**48**) to females via the spermatophore, which acts as an AA (Fig. 34). SPME analysis showed that mated females emit **48**, not present in virgin females. Larval feeding experiments with the labelled L-phenylalanine proved that **48** is only produced by males<sup>99</sup> and taken up in the adult stage.<sup>179</sup> Behavioural assays demonstrated that mated females, but also virgins perfumed with **48**, are quickly abandoned by courting males, while females lacking **48** are more attractive.<sup>97</sup> Remating can be observed after 4–6 days.<sup>167,180</sup> Females are not actively able to control the release of the antiaphrodisiac, but the mate-refusal posture depletes their

pheromone stores.<sup>168</sup> Similar to the aphrodisiac pheromone **151**, **48** is also involved in sperm competition coherence, providing information on female mating history and allowing males to adjust their composite of fertilizing and non-fertilizing sperm. In summary, *P. napi* is using a very fine-tuned olfactory signalling system.<sup>168,181</sup>

*P. rapae* also uses **48** as an antiaphrodisiac of males, but combined with indole (**161**), which is biosynthetically formed from tryptophan. The general odour of males and females were analysed by SPME, consisting of sulcatone (**110**), nonanal (**168**), decanal (**169**), and other unknown compounds, likely **152**, as well as virgin female specific benzyl alcohol.<sup>99</sup> In contrast, *P. brassicae* uses benzyl cyanide (**162**) as AA, derived from the precursor L-phenylalanine. Benzyl cyanide (**162**) is also the male general odour, accompanied by two unknown male specific compounds.<sup>99</sup> Males cloak their mates with their own odour and turning the females into chemical mimics of males.<sup>99</sup> The parasitic wasp *Trichogramma brassicae* exploits this AA cue of *P. brassicae* and uses it to ride on a mated female to her egg-laying sites where it then parasitizes the fresh laid eggs.<sup>182</sup>

Headspace analysis of *Ascia monuste* showed (*E*)- $\beta$ -ocimene (**85**) as a male-specific compound with a yet unknown function. EAD assays yielded only responses in females while males did not react.<sup>183</sup> Females of *A. monuste phileta* emitted *p*-methoxyphenol (**163**) from their genitalia when disturbed, together with fatty acids (C<sub>16</sub>–C<sub>18</sub>) and branched hydrocarbons. It probably serves as a defence against predators or acts as a signal in courtship, but it is lacking in males.<sup>184</sup>

In the tribe Antiocharini, less is known about the function of volatile scent scale contents. Ocimene (**85**) was found to be the only main volatile component in both sexes of *Hebomoia glaucippe liukiensis* and *H. g. formosana*, displaying a higher amount in male wings compared to females.<sup>146</sup> EAD assays proved a higher response of male antennae compared to female ones for **85**, which also elicited a greater response than the main volatiles of their food plant, *Crataeva religios*, limonene (**150**) and myrcene (**159**).<sup>146</sup> Males of *Anthocharis scolymus*, lacking alar scent scales, exhibit round saccular structures connected to the socket of the ordinary scale in the wing membrane, serving as androconia.<sup>165</sup> While males of this species emit a male-specific subtle floral scent consisting of (*S*)-linalool (*S*-**81**) and (*E*)- $\beta$ -farnesene (**164**), together with the minor components **168** and **169**, females are scentless (Fig. 35).<sup>165</sup> The amount of both terpenes increases in males reaching a plateau with sexual maturation after two days past eclosion. In contrast, the aldehydes reach a constant amount after only four days.<sup>165,185</sup> Terpenoids (*S*)-**81** and **164** have not been reported from other pierids, but are widespread plant constituents mediating various plant interactions, and act as insect pheromones.<sup>165,186</sup>

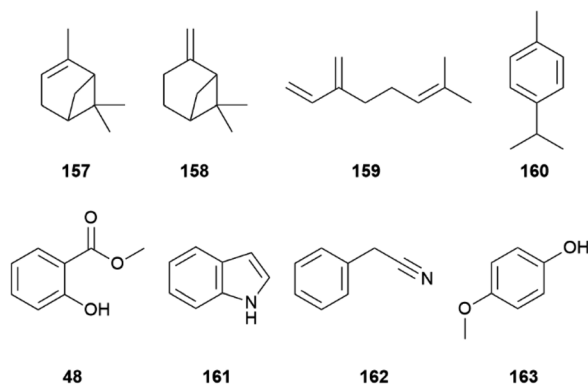


Fig. 34 Monoterpenes found in wing scent scales and antiaphrodisiacs of male *Pieris* (**48**–**162**) as well as phenol **163** emitted by female *Ascia monuste*.

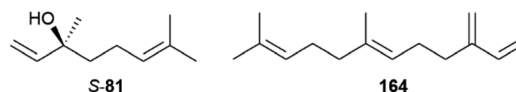


Fig. 35 Male-specific compounds of *Anthocharis scolymus*.



In summary, the volatile compounds of pierids are predominately hydrocarbons and terpenoids, next to some small amino acid derivatives and carotenoid degradation products. Within the terpenes, oxidatively degraded sesqui- or diterpenoids dominate. *P. brassicae* can take up (*E*)-phytol (154) in their diet at the larval stage and transform it into hexahydrofarnesyl acetone (117) by oxidative degradation, demonstrated by feeding experiments with deuterium labelled 154.<sup>134</sup> This is in contrast to Orchid bees who can only obtain 117 through uptake from plants.<sup>187</sup> Phytol (154) occurs in all plants as a side chain of chlorophyll A that is taken up during larval feeding, degraded during digestion to 154, and finally accumulated in scent scales of male wings, where it gets in a part oxidized to 117.<sup>134</sup> Ferrulactone (152) has been shown to be biosynthesized, formed from farnesol by the beetle *C. ferrugineus*<sup>188</sup> through oxidation and cleavage of the terminal double bond. Very likely this is also the case in *Pieris*, suggesting a similar biosynthesis of brassicalactone (153) from geranylgeraniol.

Most of the pierid compounds are not specific to these butterflies, as they are known from other natural sources, such as the abundant ketone 117. More specific compounds are 153 and 152. The latter can be conveniently synthesized starting from geranyl acetate *via* 10-hydroxy-4,8-dimethyl-4,8-decadienoic acid, followed by macro-lactonization,<sup>106,189</sup> but other methods have also been employed. Various isomers of the larger lactone 153 were synthesized using ring-closing-metathesis as a key step, leading to mixtures of *E/Z*-isomers that allowed unambiguous establishment of the double bond geometries.<sup>106</sup>

## 7 Miscellaneous species

In addition to the already discussed subfamilies of the Nymphalidae, the viceroy butterfly *Limenitis archippus* of the Limenitidinae is a Müllerian mimic to *Danaus plexippus* and *D. gilippus* and sequesters non-volatile defensive compounds, phenolic glycosides from their larval host-plant *Salix caroliniana*. When disturbed both sexes secrete benzaldehyde (165), benzoic acid (32), methyl salicylate (48) and salicylaldehyde (166) from their abdomen, probably in defence (Fig. 36).<sup>190</sup> Apart

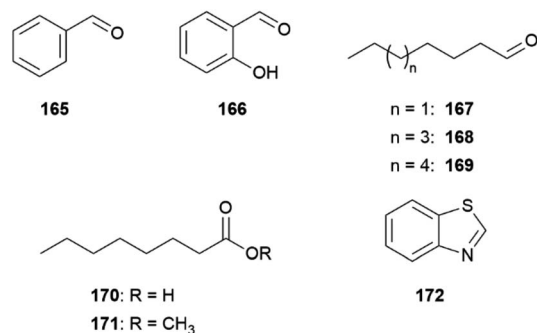


Fig. 36 Volatiles of *Limenitis archippus*, *Erynnis montanus* and other species.

from 48, these compounds are also chemical defences of willow-feeding leaf beetles against generalist predators.<sup>191</sup>

Less is known about the use of volatile compounds in the family of Hesperidae. In the species *Erynnis montanus*, part of the subfamily Pyrginae, males display a characteristic pungent odour which changes within the period of adult occurrence. Whole body extracts revealed docosane and heneicosane as the most abundant compounds in both sexes, likely representing the cuticular hydrocarbons.<sup>192</sup> Other constituents are heptanal (167), nonanal (168), decanal (169) and acetic acid, also identified in Papilionidae,<sup>193,194</sup> as well as octanoic acid (170, Fig. 36). In moderate quantities, *p*-cresol (34) is present in males, nonanoic acid in females and benzothiazole (172) in both sexes. The characteristic male compound 34 decreases with time. Both aromatics 34 and 172 have been reported as effective repellents against predatory ants.<sup>195</sup>

In the subfamily Papilioninae of the family Papilionidae, the swallowtail butterflies, terpenoids have been identified as male-specific volatiles. Males of *Papilio machaon hippocrates* emit a fragrant smell consisting of linalool (81) and geranylacetone (173) as major components, emanating from their wings. Both sexes respond in EAD assays to 81, limonene (150), nonanal (168) and methyl octanoate (171), the latter being body specific.<sup>196</sup> Also in *P. polytes* and *P. protenor demetrius* the male scent contain small amounts of 81.<sup>194,197</sup> Furthermore, a sexual dimorphism in the volatile composition in adults can be observed, for instance in *P. protenor demetrius* eight out of seventeen major compounds show significant sex differences.<sup>194,197</sup> 2,3-Butanediol (174) and 81 are present in significantly larger amounts in males than females, while 167, 168, 48, benzyl alcohol and 32 are shared by both sexes.<sup>197</sup> In *P. polytes* (*Z*)-7-tricosene (176) and dodecanoic acid are more abundant in males, and (*Z*)-7-pentacosene (177) and heptacosane in females (Fig. 37).<sup>194</sup> All compounds were found to be more concentrated on the wings than the body except for acetoin (175), which evokes a higher response in females in EAD assays.<sup>194,196</sup> Males of *P. polytes* use cuticular hydrocarbons for mate discrimination

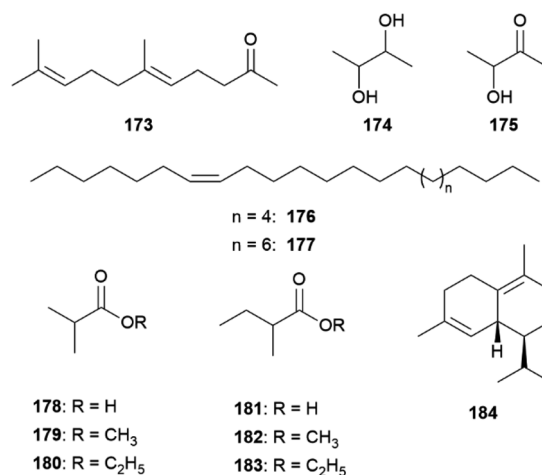


Fig. 37 Volatile compounds present in *Papilio* species (173–175) and osmeterial constituents (178–184) of Papilionidae.



to distinguish non-mimetic females from conspecific males and sympatric sister species, certain alkenes serving as behavioural inhibitors of male copulation.<sup>198</sup>

In contrast, adults of both sexes of *Luehdorfia japonica* (Parnassiinae) display a common odour and are lacking male-specific volatiles. Although most volatile compounds, like **167–169**, **165**, *p*-cymene (**160**), **150** and dodecane have been previously reported in adult scent of Papilioninae, *L. japonica* exhibits a unique chemical scent composition with limonene (**150**) being the most abundant compound.<sup>199</sup>

Additionally, larvae of Papilionidae possess a specialized defensive gland located mid-dorsally behind the head, the osmeterium. This normally invisible gland is extruded when the larvae is irritated and produces a defensive secretion, regarded as defensive allomone.<sup>200,201</sup> Species of this family can be separated into two groups. The osmeterial composition of the homogeneous type displays no quantitative alterations between instars,<sup>193,202</sup> whereas in the heterogeneous type the last instar differs remarkably from younger larvae, displaying a shift from mono- and sesquiterpenoids to aliphatic acids and their esters.<sup>200,203–206,207</sup> Regardless of the group, mono- and sesquiterpene hydrocarbons were found to be biosynthesized *de novo* from acetate precursors by larvae.<sup>208</sup> The heterogeneity of the osmeterial chemistry in the tribe Papilionini is assumed to represent a fine-tuning of the chemical defence in response to shifting predation pressures as the larvae age and grow.<sup>203</sup> While terpene secretions discourage attacks by ants, acid production is rendering the caterpillars less palatable.<sup>204</sup> In several species of the heterogeneous group the fifth instar secretion was found to contain 2-methylpropanoic acid (**178**) and 2-methylbutanoic acid (**181**) as major constituents, together with their methyl (**179**, **182**) and ethyl esters (**180**, **183**, Fig. 37).<sup>193,200,202,206,209</sup> Terpenoids, among others  $\alpha$ -pinene (**157**), which was found to be the most toxic terpenoid compound,<sup>210</sup>  $\beta$ -myrcene (**159**), limonene (**150**) and  $\delta$ -cadinene (**184**), appear as main components in different species.<sup>193,200,202,205</sup>

In the family Lycaenidae alar androconia are frequently observed on wings of many species but there is little knowledge of their scent chemistry.<sup>211,212</sup> Males of *Lycaeides argyrognomon* emit  $\delta$ -cadinol (**185**) from their androconial scales, together with **168** and hexadecyl acetate (**52**). The sesquiterpene alcohol **185** was found to be involved in the inhibition of the mate-refusal behaviour in females.<sup>213</sup> In *Celastrina argiolus lado-nides*, however, males emit a faint odour mainly composed of lavender lactone (**186**) and  $\delta$ -decalactone (**187**) from androconial battledore scales, present on the fore- and hindwings (Fig. 38). While for lactone **187** only the *R*-enantiomer is present,

**186** was found as a mixture of the *R/S*-enantiomers.<sup>212</sup> The functions of these lactones are not known yet, but may also serve a similar role as **185** in *L. argyrognomon* or indicate male identity or age for sexual selection by females.<sup>212,213</sup>

The sister families Lycaenidae and Riodinidae are also known for their larval ant associations, ranging from mutualist partners to parasites.<sup>214</sup> Some myrmecophilous butterfly larvae use chemical mimicry and concealment by chemical camouflage based on cuticular hydrocarbons.<sup>215</sup>

## 8 Conclusions

The chemistry of male butterflies is diverse and involves compounds either by *de novo* biosynthesis, or by uptake from precursors from plants, addressing various biosynthetic pathways, such as fatty acid biosynthesis, terpene and apocarotenoid formation, alkaloid modification, or aromatic compounds. Alongside compounds often or occasionally found in other natural systems, such as plants or bacteria, unique compounds are also present. They serve a multitude of fine-tuned purposes, many of which are still unknown. Some general traits can be observed in the different families discussed here. While Danaini use PA-precursors, but also use a wide variety of other, sometimes unique, compounds, the Ithomiini rely mostly on PA-derivatives with few exceptions. In the Heliconiinae, high variations in compound composition and a large number of metabolites can be observed, often preferring fatty acid derivatives or terpenoids, enabling species radiation and influence by mimicry groups. Esters play a dominant feature here. In Pierids, the compounds are often more simple, again aliphatic esters and simple terpenes, although terminal oxidation can lead to specific compounds such as macrolactones. In Satyrinae, relative simple fatty acid derivatives and esters dominate. In all families degradation products of phytol and chlorophyll, such as **68** and **159**, are used.

## 9 Author contributions

Both authors worked together on all aspects of the manuscript.

## 10 Conflicts of interest

There are no conflicts to declare.

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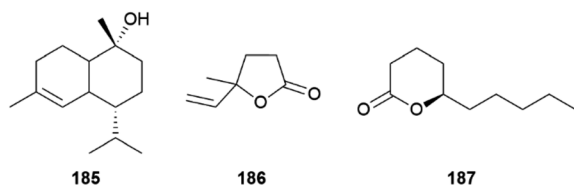
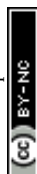


Fig. 38 Male specific androconial compounds found in Lycaenidae.





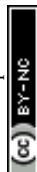
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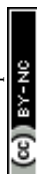
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